STRATEGIES IN THE DESIGN OF ANTIVIRAL DRUGS

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A decade ago, just five drugs were licensed for the treatment of viral infections. Since then, greater understanding of viral life cycles, prompted in particular by the need to combat human immunodeficiency virus, has resulted in the discovery and validation of several targets for therapeutic intervention. Consequently, the current antiviral repertoire now includes more than 30 drugs. But we still lack effective therapies for several viral infections, and established treatments are not always effective or well tolerated, highlighting the need for further refinement of antiviral drug design and development. Here, I describe the rationale behind current and future drug-based strategies for combating viral infections.

VIRION
A mature infectious virus particle.

Effective vaccines have led, or might lead, to the eradication of important viral pathogens, such as smallpox, polio, measles, mumps and rubella. But other viral diseases, particularly human immunodeficiency virus (HIV) and hepatitis C virus (HCV), have so far proved to be intractable to the vaccine approach. The need for effective antiviral drugs is further emphasized by the lack of vaccines for most respiratory-tract virus infections (adenovirus, rhinovirus, parainfluenza virus and respiratory syncytial virus (RSV)), the widely occurring human papilloma viruses (HPV) and herpesviruses (herpes simplex virus types 1 and 2 (HSV-1, -2), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesviruses types 6, 7 and 8 (HHV-6, -7, -8)), and the vast array of haemorrhagic fever viruses. And although vaccines have been developed for hepatitis B virus (HBV) and influenza virus types A and B, their use has not eliminated the need for effective chemotherapeutic agents.

Many new antiviral drugs have been licensed in recent years (TABLE 1), most of which are used for the treatment of HIV infections; of the current repertoire of more than 30 drugs, 16 are anti-HIV, 5 are anti-CMV, 5 are anti-HSV and anti-VZV, 1 is anti-RSV, 3 are anti-hepatitis and 4 are anti-influenza[1]. But there is considerable room for improvement, as these compounds are not always efficacious or well tolerated. The emergence of viral resistance to drugs and drug-related side effects are among the main reasons for further refinement of antiviral drug design and development.

Antiviral drug design could, in principle, be targeted at either viral proteins or cellular proteins. The first approach is likely to yield more specific, less toxic compounds, with a narrow spectrum of antiviral activity and a higher likelihood of virus drug-resistance development, whereas the second approach might afford antiviral compounds with a broader activity spectrum and less chance of resistance development, but higher likelihood of toxicity. Both routes are worth exploring, the preferred route being dictated by both the nature of the virus and the targets that the virus or its host cell have to offer.

As exemplified for HIV (FIG. 1), the viral life cycle encompasses several crucial steps, starting with the attachment of the virus to the cell and finishing with the release of the progeny VIRIONS from the cell. The replicative cycle of retroviruses, such as HIV, becomes closely associated with the host cell; after reverse transcription (RNA→DNA), the resulting proviral DNA becomes integrated into the cellular genome and then follows the 'classical' transcription and translation processes. By contrast, 'normal' cytolytic viruses, such as herpesviruses, replicate their genome and express their genes autonomously, independent of the host cell metabolism. Here, I focus primarily on approaches targeted at specific processes in viral infection (FIG. 1), including virus adsorption, virus–cell fusion, viral

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Table 1 | **The antiviral repertoire**

Approach	Target virus(es)	Compounds approved	Selected compounds in development for the indicated target virus
Virus adsorption inhibitors	HIV, HSV, CMV, RSV and other enveloped viruses		Polysulphates, polysulphonates, polycarboxylates, polyoxometalates, chicoric acid, zintevir, cosalane derivatives, negatively charged albumins
Virus–cell fusion inhibitors	HIV, RSV and other paramyxoviruses		HIV: AMD3100, TAK779 and T20 derivatives
Viral DNA polymerase inhibitors	Herpesviruses (HSV-1, -2, VZV, CMV, EBV, HHV-6, -7, -8)	Acyclovir, valaciclovir, ganciclovir, valganciclovir, penciclovir famciclovir, brivudin*, foscarnet	Bicyclic furopyrimidine nucleoside analogues, A5021, cyclohexenylguanine
Reverse transcriptase inhibitors	HIV	NRTIs: zidovudine, didanosine, zalcitabine, stavudine, lamivudine‡, abacavir NNRTIs: nevirapine, delavirdine, efavirenz	Emtricitabine, amdoxovir Emivirine, UC781, DPC083, TMC125 (R165335)
Acyclic nucleoside phosphonates	DNA viruses (polyoma-, papilloma-, herpes-, adeno- and poxviruses), HIV, HBV	CMV: cidofovir HIV: tenofovir	HBV: adefovir
Inhibitors of processes associated with viral RNA synthesis	HIV, HCV		
Viral protease inhibitors	HIV, herpesviruses, rhinoviruses, HCV	HIV: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir	HIV: atazanavir, mozenavir, tipranavir Human rhinovirus: AG7088
Viral neuraminidase inhibitors	Influenza A and B virus	Zanamivir, oseltamivir§	RWJ270201
IMP dehydrogenase inhibitors	HCV, RSV	Ribavirin‖	Mycophenolic acid, EICAR, VX497
S-adenosylhomocysteine hydrolase inhibitors	(–)RNA haemorrhagic fever viruses (for example, Ebola)		

* Brivudin is approved in some countries; for example, Germany.
‡ Lamivudine is also approved for the treatment of HBV.
§ In addition to zanamivir and oseltamivir, amantadine and rimantadine have been approved as anti-influenza drugs, but these compounds are targeted at the viral uncoating process, not the viral neuraminidase.
‖ Ribavirin is used in combination with interferon-α for HCV.
CMV, cytomegalovirus; EBV, Epstein–Barr virus; EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IMP, inosine 5′-monophosphate; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

DNA or RNA synthesis (although host cellular components are also associated with these processes), and viral enzymes, such as protease and neuraminidase. Two host cellular enzymes — inosine 5′-monophosphate (IMP) dehydrogenase and *S*-adenosylhomocysteine (SAH) hydrolase — could also be targets for certain classes of antiviral agents.

Virus adsorption inhibitors

Numerous polyanionic compounds (for example, polysulphates such as polyvinylalcohol sulphate, polysulphonates such as polyvinylsulphonate (FIG. 2), polycarboxylates, polynucleotides such as zintevir, polyoxometalates and negatively charged albumins) have been shown to inhibit HIV replication by preventing virus attachment (adsorption) to the surface of the host cell. All of these negatively charged polymers might be expected to interact with the positively charged amino acids in the V3 LOOP of the HIV glycoprotein, gp120, which is rich in arginine (R) and lysine (K) residues. In doing so, the polyanions shield the V3 loop and therefore hamper the binding of the HIV virions to heparan sulphate[2], the primary binding site at the cell surface, before more specific binding occurs with the CD4 receptor on $CD4^+$ cells.

Heparan sulphate is widely expressed on animal cells, and is involved in virus–cell binding for a broad array of ENVELOPED viruses, including HSV[3] and dengue virus[4]. So, polysulphates, polysulphonates and other polyanionic substances that interfere with the binding of these enveloped viruses to target cells could be effective in both the treatment and prevention of such infections. Polyanionic substances could be important as vaginal microbicides as, in an appropriate formulation, they might prevent sexual transmission of HIV infection. Moreover, these polyanions are not only active against HIV, but also against HSV and other sexually transmitted disease (STD) pathogens, such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*[5].

Although polyanions might have several sites of interaction, virus–cell attachment would be the preferred target from a therapeutic viewpoint, as it is the first opportunity to curtail the viral life cycle, and the polyanions do not need to enter the cells, which would be problematic. The interaction of polyanionic substances at this level can also be considered specific, as repeated passage of the HIV virus in the presence of polyanions can lead to resistance mediated by mutations in the envelope glycoprotein gp120, particularly in the V3 loop (K269E, Q278H, N293D), as originally shown for dextran sulphate[6], and subsequently for zintevir[7] and negatively charged albumins[8].

V3 LOOP
The gp120 protein has eleven defined loop segments, five of which are termed variable (designated V1–V5).

ENVELOPE
A lipoprotein-bilayer outer membrane of many viruses. Envelope proteins often aid in identifying and attaching the virus to a cell-surface receptor, whereby viral entry can occur.

Virus–cell fusion inhibitors

Enveloped viruses, as a rule, enter their host cells by fusion between the viral envelope and cellular plasma membrane (FIG. 1). This fusion process is basically similar for several enveloped virus families (that is, retro-, paramyxo- and herpesviruses), but for HIV it is preceded by the interaction of gp120 with its co-receptor on the host cell — the chemokine (C-X-C) motif receptor 4 (CXCR4) for T-tropic or X4 HIV strains, or the chemokine (C-C) motif receptor 5 (CCR5) for M-tropic or R5 HIV strains. CXCR4 and CCR5 normally act as the receptors for the C-X-C chemokine, SDF1 (stromal-cell-derived factor 1), and the C-C chemokines RANTES (regulated upon activation, normal T-cell expressed and secreted) and MIP1 (macrophage inflammatory protein 1), respectively. The coincidental use of both CXCR4 and CCR5 by HIV as co-receptors to enter cells has prompted the search for CXCR4 and CCR5 antagonists, which, through blockade of the corresponding co-receptor, might be able to block HIV entry into the cells.

This has now been shown with several compounds, the most prominent among the CXCR4 antagonists being the bicyclam AMD3100 (REFS 9,10 and FIG. 2), and the best documented among the CCR5 antagonists being TAK779 (REFS 11,12 and FIG. 2). The site of interaction of TAK779 with the transmembrane helices of CCR5 has been mapped[12] (FIG. 3), and, likewise, crucial amino-acid residues involved in the binding of AMD3100 to CXCR4 have been identified[13]. Recently, a new CCR5 antagonist, SCH-C (SCH351125), was announced as an orally bioavailable inhibitor of M-tropic R5 strains that is capable of suppressing R5 HIV-1 infection both *in vitro* and *in vivo* (SCID-hu Thy/Liv mice)[14]. The clinical potential of the CXCR4 and CCR5 antagonists in the management of HIV infections remains to be proved. To ensure maximal coverage of both X4 and R5 strains, dual CXCR4/CCR5 antagonists should be developed, or single CCR5 and CXCR4 antagonists should be combined.

The interaction of gp120 with its co-receptor (CCR5 or CXCR4) triggers a series of conformational changes in the gp120–gp41 complex that ultimately lead to the formation of a 'trimer-of-hairpins' structure in gp41 — a bundle of six α-helices: three α-helices formed by the carboxy-terminal regions packed in an antiparallel manner with three α-helices formed by the amino-terminal regions. The fusion-peptide region, located at the extreme amino terminus, will insert into the cellular membrane, whereas the carboxy-terminal region remains anchored in the viral envelope. In this sense, the trimer-of-hairpins motif brings the two membranes together, so agents that interfere with the formation of the gp41 trimer-of-hairpins structure might be expected to inhibit the fusion process[15].

Several constructs have been designed to interfere with the gp41-mediated fusion process: the so-called '5-helix', which binds the carboxy-terminal region of gp41 (REF. 15); D-peptide inhibitors, which dock into the pocket formed by the α-helices of gp41 (REF. 16); and T20 (pentafuside, previously called DP178, a synthetic 36-amino-acid

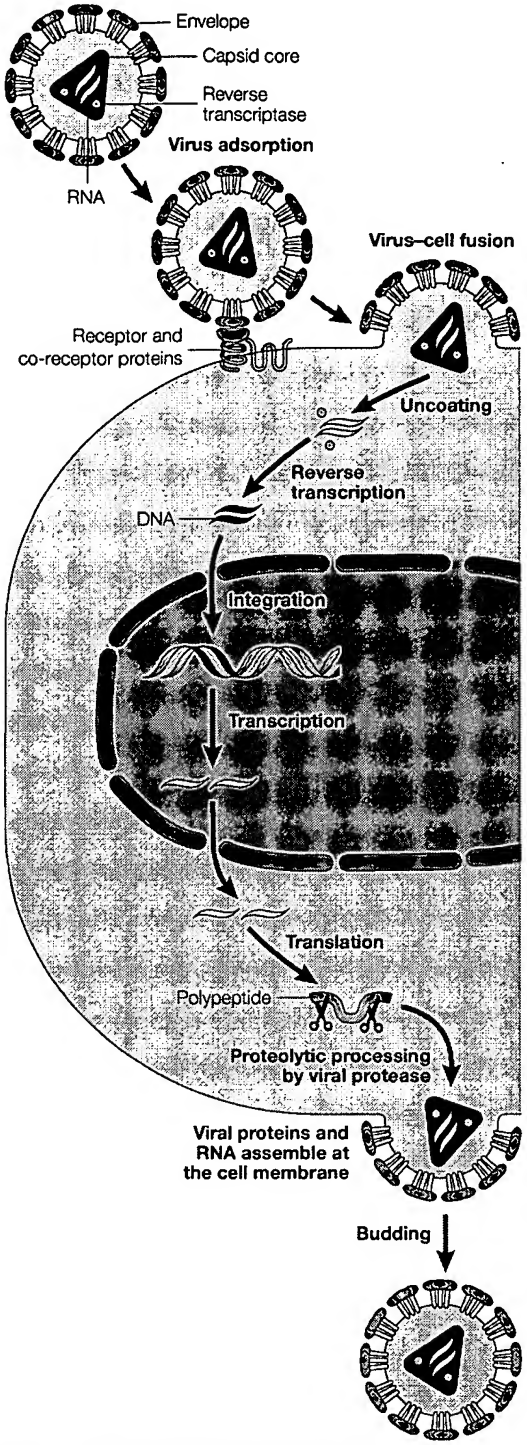

Figure 1 | **The viral life cycle, as exemplified by HIV.** Viral life cycles have several specific steps, many of which are targets for antiviral drugs. After virus adsorption, enveloped viruses enter the cell by virus–cell fusion. For human immunodeficiency virus (HIV), which is a retrovirus with an RNA (yellow) genome, replication of the genome occurs after reverse transcription and integration into the host cell chromosome. For DNA viruses, such as herpesviruses, the genome is replicated by a viral DNA polymerase. After transcription to RNA, followed by translation and proteolytic processing of the precursor polypeptide, viral proteins assemble at the cell membrane, from which they bud to release new virions.

Animated online

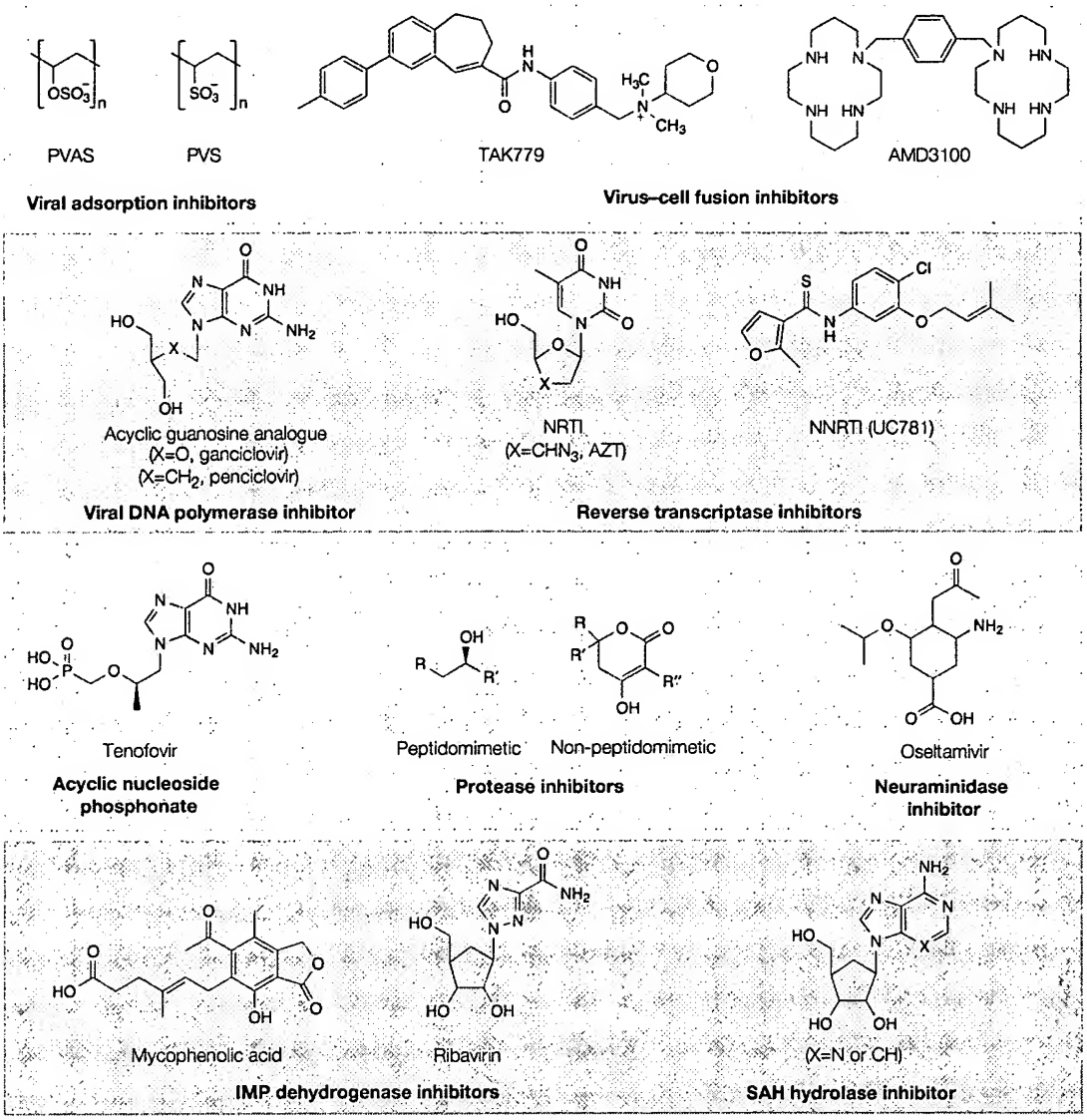

Figure 2 | **Basic (skeletal) pharmacophores or prototypic compounds of the classes of antiviral agents described in this review.** AZT, azidothymidine; IMP, inosine monophosphate; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PVAS, polyvinylalcohol sulphate; PVS, polyvinylsulphonate; SAH, *S*-adenosylhomocysteine.

peptide that corresponds to residues 127–162 of the ectodomain of gp41). T20 has proved effective in reducing plasma HIV levels in humans, providing the proof of concept that viral entry can be successfully blocked *in vivo*[17].

Insight into the HIV fusion process should help in designing fusion inhibitors for other viruses as well, as trimer-of-hairpins motifs could also be predicted for other virus families[15], including paramyxoviridae, such as parainfluenza virus, measles and respiratory syncytial virus. In fact, for each of these paramyxoviruses, peptides similar to T20 have been shown to block viral fusion[18]. Also, a cobalt-chelating complex (CTC96) that inhibits infection by HSV-1 through blocking fusion[19] might have an extended antiviral activity spectrum, given the premise that enveloped viruses belonging to different families share an analogous process of membrane fusion.

PRODRUG
A pharmacologically inactive compound that is converted to the active form of the drug by endogenous enzymes or metabolism. It is generally designed to overcome problems associated with stability, toxicity, lack of specificity or limited (oral) bioavailability.

Inhibitors of viral DNA or RNA synthesis

Viral DNA polymerase inhibitors. In contrast to retroviruses (FIG. 1), herpesviruses do not have a reverse transcription step in their replicative cycle, which means that their DNA genome can be replicated by the viral DNA polymerase after the latter has been expressed in the virus-infected cell. At present, all the antiviral agents that are available for the treatment of herpesvirus infections are nucleoside analogues: either acyclic guanosine analogues (that is, acyclovir, penciclovir, ganciclovir, and their oral PRODRUG forms valaciclovir, famciclovir and valganciclovir, respectively), or thymidine analogues (brivudin) (FIG. 2 and TABLE 1). All of these compounds target the viral DNA polymerase, but before they can interact with viral DNA synthesis, they need to be phosphorylated intracellularly to the triphosphate form. The first (and, for brivudin, also the second) phosphorylation step is ensured by the HSV- or VZV-encoded

thymidine kinase, or the CMV-encoded protein kinase, and is therefore confined to virus-infected cells, which explains the specific antiviral action of the established antiherpetic compounds. Subsequent phosphorylations are achieved by host cellular kinases. In their triphosphate form, the nucleoside analogues interact with the viral DNA polymerase, either as competitive inhibitors or as alternative substrates with respect to the natural substrate (dGTP for the guanosine analogues, dTTP for the thymidine analogues). If the acyclic guanosine analogues act as alternative substrates, their incorporation prevents further chain elongation (FIG. 4a).

So, is there room for improvement? The moderate oral bioavailability of the acyclic guanosine analogues has been improved by formulating them as prodrugs. The success obtained with acyclovir, valaciclovir and famciclovir in the treatment of HSV and VZV infections has impeded further progress in this area. However, brivudin, which is considerably more potent than acyclovir and penciclovir as an anti-VZV agent, represents an important alternative for the treatment of VZV infections. Furthermore, although brivudin is active in the nanomolar concentration range against VZV replication, its potency can be superseded by bicyclic furopyrimidine nucleoside analogues bearing a long alkyl or alkylaryl side chain attached to the furane ring[20,21]. The mechanism of action of these exquisitely potent and selective anti-VZV agents remains to be elucidated, although there is no doubt that their specificity for VZV is governed by the virus-encoded thymidine kinase.

In the guanosine analogue class, several new CONGENERS have been described; namely A5021 (REFS 22,23) and the D- and L-enantiomers of cyclohexenylguanine[24]. These compounds seem to have an activity spectrum and mode of action similar to that of acyclovir, but further studies are warranted to verify whether these new guanosine analogues might have an extended spectrum of activity (that is, against HHV-6, -7 and -8, which are not particularly sensitive to acyclovir), increased *in vivo* efficacy, or improved pharmacokinetics.

Reverse transcriptase inhibitors. As is evident from FIG. 1, reverse transcriptase is essential in the replicative cycle of retroviruses, such as HIV, as it synthesizes the proviral DNA, which will then be integrated into the host cell genome and passed on to all of the progeny cells. The substrate (dNTP) binding site of HIV reverse transcriptase (RT) has proved to be an attractive target for nucleosidic HIV inhibitors: six nucleoside analogues — zidovudine (azidothymidine, AZT), didanosine (dideoxyinosine), zalcitabine (dideoxycytidine), stavudine (didehydrodideoxythymidine, d4T), lamivudine (3′-thiadideoxycytidine) and abacavir — have been licensed as anti-HIV drugs (FIG. 2 and TABLE 1), and several others, such as emtricitabine and amdoxovir, are in advanced development. All of these dideoxynucleoside analogues act according to a similar 'recipe', as exemplified for AZT (FIG. 4b). They must be phosphorylated consecutively inside the host cell by three cellular kinases — a nucleoside kinase, a nucleoside 5′-monophosphate kinase and a nucleoside 5′-diphosphate kinase — to form the corresponding 5′-triphosphate derivative, which can interact, as a chain terminator, with the reverse transcription (RNA→DNA) reaction. One of the mechanisms by which resistance to AZT might arise is through removal of the chain-terminating residue, a kind of repair reaction involving pyrophosphorolysis, which can be regarded as the opposite of the reverse transcriptase reaction. Not all chain terminators are readily removed; for example, the acyclic nucleoside phosphonate derivative tenofovir (PMPA; FIG. 2) is not, and, in this sense, PMPA should be less prone to resistance development than the regular nucleoside analogues.

The first phosphorylation step that converts the 2′,3′-dideoxynucleoside analogues to their 5′-monophosphate can be considered as the bottleneck in the overall metabolic pathway leading to the formation of the active 5′-triphosphate metabolites. If certain dideoxynucleoside analogues (for example, 2′,3′-dideoxyuridine) are not active against HIV under conditions in which others are, this stems from their poor, or lack of, phosphorylation at the nucleoside-kinase level. Therefore, attempts have been made at constructing prodrugs of 2′,3′-dideoxynucleoside 5′-monophosphate that deliver the 5′-monophosphate derivatives on cellular uptake, which can then be converted into the corresponding 5′-di- and 5′-triphosphate derivatives. This approach bypasses the initial nucleoside-kinase dependency and has been validated by the design of the phosphoramidate[25,26] and cyclosaligenyl[27,28] prodrugs of d4T 5′-monophosphate. Both prodrugs were found to deliver d4T 5′-monophosphate efficiently within the cells, which, after conversion to its 5′-triphosphate, afforded anti-HIV

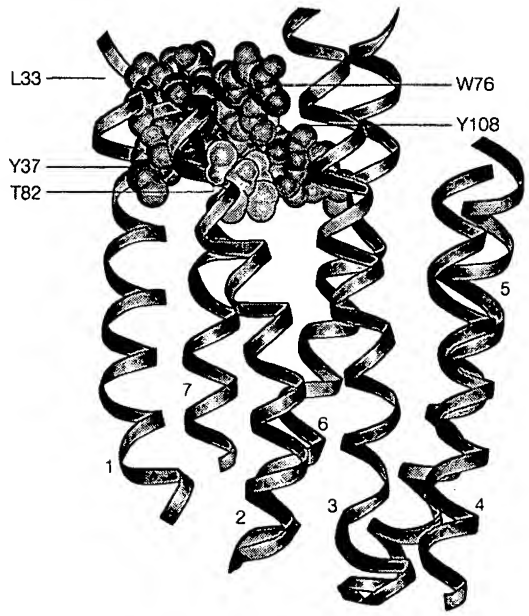

Figure 3 | **Interaction of CCR5 with TAK779.** A structural model of CCR5 complexed with TAK779 (FIG. 2), viewed from within the plane of the membrane[12]. The indicated cluster of amino acids in the TAK779 binding site includes several aromatic residues (Y37, W86, Y108) that might form favourable interactions with the aromatic rings of TAK779. (Reprinted with permission from REF. 12 © 2000, National Academy of Sciences, USA.)

CONGENER
Any member of the same chemical family.

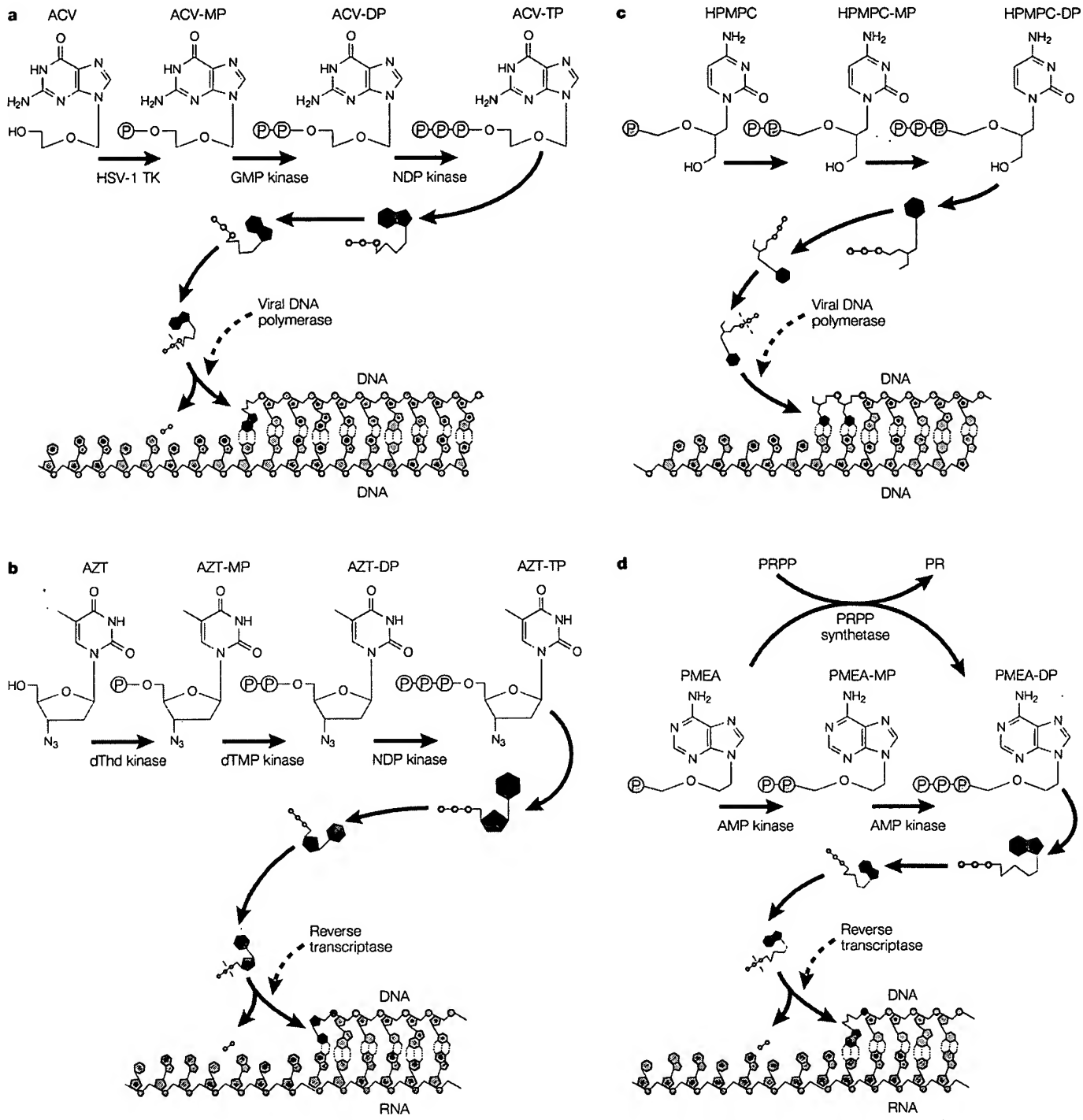

Figure 4 | **Examples of antiviral nucleoside analogues acting by a chain termination mechanism. a** | Acyclovir (ACV) targets viral DNA polymerases, such as the herpesvirus (HSV) DNA polymerase. Before it can interact with viral DNA synthesis, it needs to be phosphorylated intracellularly, in three steps, into the triphosphate form. The first phosphorylation step is ensured by the HSV-encoded thymidine kinase (TK), and is therefore confined to virus-infected cells. **b** | Azidothymidine (AZT) targets the human immunodeficiency virus (HIV) reverse transcriptase, and also needs to be phosphorylated, in three steps, to the triphospate form before it can interfere with reverse transcription. **c** | Cidofovir (S-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HPMPC), an acyclic nucleotide analogue, which can be viewed as an acyclic nucleoside analogue extended by a phosphonate moiety, targets viral DNA polymerases, and is active against DNA viruses, whether or not they encode a specific viral thymidine kinase. In contrast to acyclovir and azidothymidine, cidofovir requires only two phosphorylations to be converted to the active (triphosphate) form. **d** | Adefovir (9-(2-phosphonylmethoxyethyl)adenine; PMEA) — also an acyclic nucleoside phosphonate — is active against retroviruses and hepadnaviruses; similar to cidofovir, adefovir needs only two phosphorylations and so can bypass the nucleoside-kinase reaction that limits the activity of dideoxynucleoside analogues such as AZT. DP, diphosphate; dThd, (2'deoxy)-thymidine; MP, monophosphate; NDP, nucleoside 5'-diphosphate; PR, 5-phosphoribose; PRPP, 5-phosphoribosyl-1-pyrophosphate; TP, triphosphate.

activity under conditions in which the d4T nucleoside, owing to inefficient phosphorylation, did not. It remains to be established whether this nucleoside-kinase bypass strategy will also yield increased antiviral efficacy *in vivo*.

All of the aforementioned 2',3'-dideoxynucleoside analogues, in their 5'-triphosphate form, act as competitive substrates/inhibitors with respect to the natural substrates (dNTPs) at the catalytic site of HIV RT, and, as HBV uses a similar RT in its life cycle, these compounds might also be able to inhibit HBV replication. This premise has been borne out in particular for lamivudine, which is licensed for the treatment of chronic HBV infections.

Such an extended activity spectrum cannot be anticipated for a second class of RT inhibitors — the non-nucleoside reverse transcriptase inhibitors (NNRTIs) — which interact with an ALLOSTERIC, non-substrate binding ('pocket') site on HIV-1 RT[29]. This 'pocket' does not exist in ligand-free RT, and does not occur in RTs other than HIV-1 RT, or, if it does, only the HIV-1 RT pocket offers the necessary interactions with NNRTIs. These interactions are: stacking interactions with the aromatic amino acids Y181, Y188, W229 and Y318; electrostatic interactions with K101, K103 and E138; van der Waals interactions with L100, V106, Y181, G190, W229, L234 and Y318; and hydrogen bonding with the main-chain peptide bonds[30]. A model for the interaction of a representative NNRTI, the thiocarboxanilide UC781 (FIG. 2), with HIV-1 RT[31] is shown in FIG. 5.

NNRTIs are notorious for rapidly eliciting virus resistance resulting from mutations at amino-acid residues that surround the NNRTI-binding site. In the clinic, the most prominent mutations engendering resistance to NNRTIs are the K103N and Y181C mutations. At present, only three NNRTIs (nevirapine, delavirdine and efavirenz) have been formally licensed, although several others are in the developmental stage. However, it is obvious that in the future design of new NNRTIs, not only potency and safety, but also resilience to drug-resistance mutations should be taken into account[32]. It is noteworthy that some amino acids that surround the NNRTI-binding site, such as W229 and Y318, do not seem apt to mutate, or, if they do, they lead to a 'suicidal' loss of RT activity[33]. Such immutable amino acids could be prime targets for the rational design of new NNRTIs.

Acyclic nucleoside phosphonates. The acyclic nucleoside phosphonates can be viewed as acyclic nucleoside analogues that are extended by a phosphonate moiety. The phosphonate group is equivalent to a phosphate group, but, unlike phosphate, phosphonate can no longer be cleaved by the esterases that would normally convert nucleoside monophosphates back to their nucleoside form. Consequently, acyclic nucleoside phosphonates might show a broadened antiviral activity spectrum compared with those of acyclic nucleoside analogues such as acyclovir, and dideoxynucleoside analogues such as zidovudine. On the one hand, they should be active against those DNA viruses that do not encode a specific viral thymidine kinase (TK) or protein kinase (PK), or that have become resistant to the nucleoside analogues through TK or PK deficiency. On the other hand, they should also be able to bypass the nucleoside-kinase reaction that limits the activity of the dideoxynucleoside analogues against retroviruses such as HIV, and hepadnaviruses such as HBV.

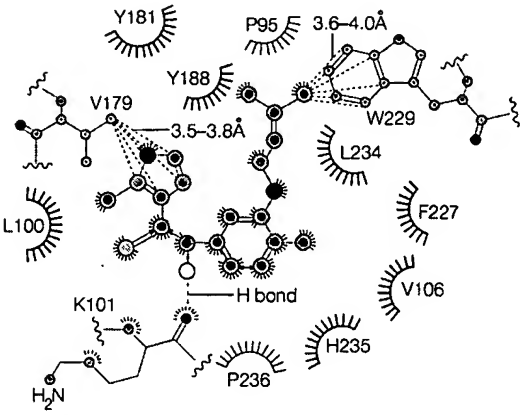

Figure 5 | **Interaction of HIV-1 RT with UC781.** Features stabilizing the complex between the human immunodeficiency virus 1 (HIV-1) reverse transcriptase (RT) and the non-nucleoside reverse transcriptase inhibitor UC781 (FIG. 2). The hydrogen bond with K101, and the two methyl-group–aromatic-ring interactions are shown explicitly. Other main hydrophobic contacts are shown with bold lines; minor ones are shown with faint lines[31]. Standard CPK COLOURING is used.

These objectives have been fulfilled on both scores, albeit by different types of acyclic nucleoside phosphonate: cidofovir (HPMPC) has broad-spectrum activity against DNA viruses; and adefovir (PMEA) and tenofovir (PMPA) have activity against retro- and hepadnaviruses.

Although their eventual activity spectrums are different, both types of compound share a common strategy in their modes of action: they both need two (instead of three) phosphorylation steps to be converted into their active (diphosphorylated) metabolites, which then act as chain terminators in the DNA polymerase reaction (HPMPC; FIG. 4c) or reverse transcriptase reaction (PMEA, PMPA; FIG. 4d). For HPMPC to shut down viral DNA synthesis, the incorporation of two consecutive HPMPC units is required[34], whereas for PMEA, one such incorporation suffices[35]. In both cases, the acyclic nucleotides remain stably incorporated, presumably because the phosphonate group prevents repair enzymes from excising these nucleotides.

The 'era' of the acyclic nucleoside phosphonates started in 1986, with the description of the broad-spectrum anti-DNA virus activity of the adenine derivative HPMPA[36]. Its cytosine counterpart, cidofovir (HPMPC), which seemed less harmful to the host in preliminary toxicity experiments, was then developed as an antiviral drug[37], and was approved for clinical use in the treatment of CMV retinitis in AIDS patients. Cidofovir also holds great potential for the treatment of several other DNA virus infections. These include: TK-deficient HSV and VZV infections, which are resistant to acyclovir (or brivudin); herpesvirus infections, such as EBV, HHV-6, HHV-7 and HHV-8; HPV infections,

ALLOSTERIC SITES
Two or more topologically distinct binding sites within a protein can interact functionally with each other. So, two sites in different positions can bind ligands (substrates, inhibitors and so on), and binding of a ligand at one site alters the properties of the other(s).

CPK COLOURING
The CPK colour scheme for elements is based on the colours of the popular plastic space-filling models developed by Corey, Pauling and Kultun, and is conventionally used by chemists. In this scheme, carbon is represented in light grey, oxygen in red, nitrogen in blue, sulphur in yellow, hydrogen in white and chlorine in green.

including pharyngeal, oesophageal and laryngeal papillomatosis, plantar and genital warts, and cervical intraepithelial neoplasia; polyomavirus infections (progressive multifocal leukoencephalopathy); adenovirus infections (epidemic keratoconjunctivitis); and poxvirus infections[38], such as smallpox, monkeypox, cowpox, orf virus and molluscum contagiosum. Adefovir and tenofovir, the two other leading acyclic nucleoside phosphonates, have both progressed, in their oral prodrug forms, adefovir dipivoxil and tenofovir disoproxil, to Phase III clinical trials for the treatment of HBV and HIV, respectively. Tenofovir disoproxil fumarate has recently been approved in the United States for the treatment of HIV infections.

In contrast to all other antiviral drugs, acyclic nucleoside phosphonates have a particularly long intracellular half-life (1 to several days), allowing infrequent dosing (once daily for adefovir and tenofovir, or once weekly or every other week for cidofovir). Furthermore, they do not lead easily to resistance, even after prolonged treatment for more than one or two years. No drug metabolic interactions are known for the acyclic nucleoside phosphonates, which means that they can readily be added to any drug regimen, as has been shown in particular for tenofovir in the treatment of HIV infections.

Inhibitors of associated processes. Gene expression (that is, transcription to viral RNA) of the genome of retroviruses, such as HIV, is not possible without integration of the proviral DNA into the host chromosome (FIG. 1). So, the enzyme involved — integrase — has been considered an attractive target for chemotherapeutic intervention. Numerous integrase inhibitors have been described[39,40], although none has sufficient specificity to be further pursued as an integrase-targeted drug. The problem with integrase inhibitors is that, although they might be effective in enzyme-based assays, their anti-HIV activity in cell culture can be masked by cytotoxicity. And even if selective anti-HIV activity in cell culture is noted, caution should be exercised in unconditionally attributing this activity to inhibition of the integration process, as the compounds concerned might owe their anti-HIV activity to an action targeted elsewhere. This has proved to be the case for the anionic compounds zintevir[7] and L-chicoric acid[41], two integrase inhibitors that owe their anti-HIV activity primarily to an interaction with the viral envelope protein gp120, and so fall into the category of the polyanionic inhibitors of virus adsorption discussed above. At present, the only compounds that qualify as genuine integrase inhibitors are the diketo acids L731,988 and L708,906 (REFS 42,43). In cell culture, these compounds were shown to inhibit both the replication of HIV-1 and the strand transfer function of the integrase (the other catalytic function of the enzyme being endonucleolytic cleavage of the terminal dinucleotide GT from the 3' end of the substrate DNA). Furthermore, these two events could be causally linked, as mutations in the HIV-1 integrase conferred resistance to the inhibitory effects of the compounds on both strand transfer and HIV-1 infectivity[42].

At the transcription level, HIV gene expression could be inhibited by compounds that interact with cellular factors that bind to the long terminal repeat (LTR) promoter, and which are needed for basal-level transcription, such as NF-κB inhibitors[44]. However, greater specificity might be achieved using compounds that specifically inhibit the transactivation of the HIV LTR promoter by the viral *trans*-acting transactivator (tat) protein. The tat protein interacts specifically with the tat responsive element, which is located at the beginning of the viral messenger RNA that is transcribed from the LTR promoter, thereby enhancing the transcription process. Several compounds have been described as inhibitors of the transcription process; for example, fluoroquinolines[45], and bistriazoloacridone derivatives, such as temacrazine[46]. The latter was found to block HIV-1 RNA transcription that starts from the HIV LTR promoter, without interfering with the transcription of any cellular genes. The peptide analogue CGP64222, which is structurally reminiscent of amino acids 48–56 (RKKRRQRRR) of the tat protein, was also designed to act as a tat antagonist[47]. However, although CGP64222 is able to interact with the tat-driven transcription process, its anti-HIV activity in cell culture is mediated primarily by an interaction with CXCR4, the co-receptor for X4 HIV strains[48].

Viral RNA transcription might also be affected by targeting cyclin-dependent kinases (CDKs), which are required for the replication of many viruses, including HIV. Indeed, flavopiridol, a typical inhibitor of CDKs (in particular, CDK9, which is involved in the tat-driven transcription process), has proved to be effective in blocking HIV infectivity[49].

One of the virus infections in the greatest need of antiviral therapy is HCV. In this case, two specific enzymatic functions associated with viral RNA synthesis could be predicted to be targets for the design of new antiviral agents: first, the non-structural protein 3 (NS3)-associated helicase; and second, the non-structural protein 5B (NS5B) RNA-dependent RNA polymerase. Crystal structures of both NS5B and the NS3 helicase are available[50], and both enzymatic activities have been characterized in sufficient detail[51,52] to facilitate the development of effective HCV chemotherapeutics. For the helicase, there is no precedent, but for the RNA polymerase there is, and the experience gathered from studies of the HIV RT inhibitors might be of paramount importance when targeting the HCV RNA polymerase, especially if, as seems possible, this enzyme shows similar kinetics to the HIV RT[52].

Viral protease inhibitors

Viral proteases are crucial in the life cycle of many viruses, including retroviruses such as HIV, herpesviruses, picornaviruses such as rhinovirus, and flaviviruses such as HCV. Viral proteases have therefore been favoured as targets for antiviral agents[53]. Proteases cleave newly expressed precursor polyproteins into smaller, mature viral proteins, termed 'functional' (if endowed with enzymatic activity) or 'structural' (if part of the virion structure). For example, in HIV replication, HIV protease cleaves the glycosaminoglycan (gag) and gag–polymerase

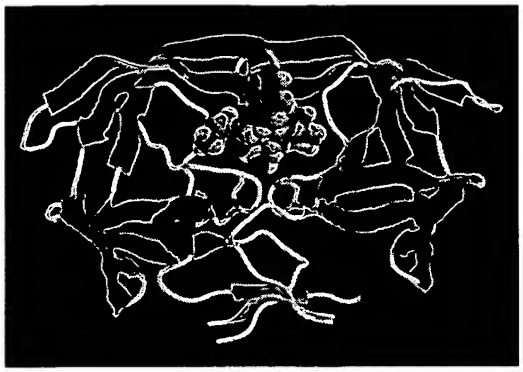

Figure 6 | **Interaction of HIV protease with KNI272.** Ribbon diagram of human immunodeficiency virus (HIV) protease complexed with the peptidomimetic protease inhibitor KNI272; derived from the crystal structure[54]. The inhibitor is shown as a space-filling model, and the two active-site aspartic acids are shown as sticks; both have standard CPK colouring.

(pol) precursor proteins to structural proteins (p17, p24, p9 and p7) and functional proteins (protease, reverse transcriptase/RNase H and integrase). HIV protease inhibitors have been tailored to the peptidic linkages (for example; F-P, F-L and F-T) in the gag and gag–pol precursor proteins that are cleaved by the protease, and have been extensively modelled in the active site of the enzyme, which is formed at the interface of two homodimeric subunits and contains two catalytic aspartic residues (each belonging to the DTG motif; FIG. 6)[54]. All of the protease inhibitors licensed at present for the treatment of HIV infection, namely saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir (TABLE 1), share the same structural determinant (FIG. 2) — a hydroxyethylene core (instead of the normal peptidic linkage) that makes them non-scissile peptidomimetic substrate analogues for the HIV protease. The HIV protease inhibitors have proved to be valuable therapeutics in combination with NRTIs and NNRTIs (a drug combination schedule known as 'highly active antiretroviral therapy' (HAART)) in the treatment of HIV infections. However, they are met by confounding factors, such as difficulties in drug adherence, drug–drug interactions, overlapping resistance patterns and long-term side effects, including lipodystrophy, cardiovascular disturbances and metabolic disturbances, such as diabetes. This has prompted the search for new, non-peptidic inhibitors of HIV protease, with cyclic urea, 4-hydroxycoumarin, L-mannaric acid or 4-hydroxy-5,6-dihydro-2-pyrone as the central scaffold instead of the peptidomimetic hydroxyethylene core[55,56] (FIG. 2); for example, tipranavir. Such compounds should show little, if any, cross-resistance with the peptidomimetic inhibitors. At present, however, their *in vivo* efficacy, pharmacokinetic profile and short- and long-term safety remain to be established.

Whether the protease-inhibitor approach would be as successful for tackling herpes-, picorna- and flaviviruses as for HIV remains to be seen. Whereas the HIV protease is an aspartate protease, herpesvirus proteases are serine proteases that have SHH as the catalytic triad[57]. Several non-peptidic inhibitors of the herpesviral protease CMV protease, which is also referred to as 'assemblin', because of its role in the CMV assembly process, have been described; for example, thieno[2,3-D]oxazinones[58], aryl hydroxylamine derivatives[59], monobactams[60], pyrrolidine-5,5-*trans*-lactams[61], 1,4-dihydroxynaphthalene and naphthoquinones[62]. Although a useful exercise in targeting the herpesviral protease, all of these efforts should be viewed as a prelude to further investigations on the *in vitro* and *in vivo* inhibitory effects of these compounds on virus replication.

Further advanced is the structure-assisted design of mechanism-based irreversible inhibitors of the human rhinovirus 3C protease — a cysteine protease involved in the proteolytic cleavage of the viral precursor polyprotein to both capsid and functional proteins required for RNA replication. This work has yielded a wealth of compounds with potent activity against several rhinovirus SEROTYPES[63–67]. One compound of the series, AG7088, which was shown to inhibit rhinovirus replication even when added late in the virus life cycle[68], has proceeded into clinical trials.

The HCV protease is a serine protease that is encoded by the non-structural NS3 domain, and is responsible for the proteolytic cleavage of the non-structural NS3, NS4A, NS4B, NS5A and NS5B proteins from the viral polyprotein (the NS4A protein then binds to the NS3 protein and enhances its proteolytic activity). The HCV NS3–4A protease is remarkably similar to the pestiviral NS3–4A protease, which is found in bovine viral diarrhoea virus (BVDV)[69], and has been intensively pursued as a target for the design of inhibitors. Again, as for the herpesvirus serine protease, several inhibitors, both peptide based[70,71] and non-peptidic[72], of the HCV NS3–4A protease have been identified, but as there is no cell-culture assay available for HCV, their activity against HCV could not be assessed. Given the similarities of the HCV and BVDV NS3–4A proteases, it seems advisable to evaluate putative HCV protease inhibitors for their activity against BVDV replication, for which cell-culture assay systems have been established.

Viral neuraminidase inhibitors

Influenza virus (both A and B) has adopted a unique replication strategy by using one of its surface glycoproteins, haemagglutinin, to bind to the target-cell receptor, which contains a terminal sialic acid. The other surface glycoprotein, neuraminidase, cleaves off the terminal sialic acid of the host cell receptor, allowing virus particles to leave the cell after the viral replicative cycle has been completed. The viral neuraminidase is therefore needed for the elution of newly formed virus particles from the cells. In addition, the viral neuraminidase might also promote viral movement through the respiratory-tract mucus, thereby enhancing viral infectivity.

So, influenza viral neuraminidase has been envisaged as a suitable target for the design of specific inhibitors. Computer-assisted drug design, based on the crystal structure of the influenza viral neuraminidase, led to the identification of zanamivir (GG167) as a specific and

SEROTYPE
Variety of a species (usually bacteria or virus) characterized by its antigenic properties.

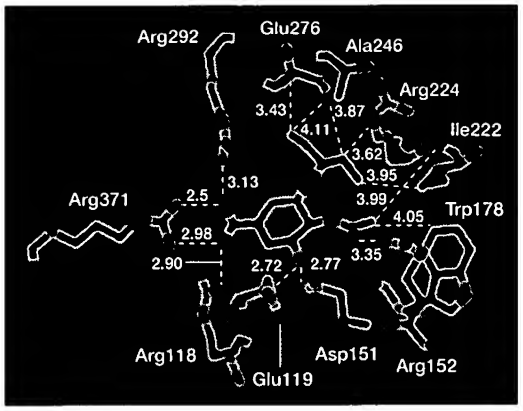

Figure 7 | **Interaction of influenza neuraminidase with oseltamivir.** Shows binding interactions of the neuraminidase inhibitor oseltamivir (FIG. 2) with influenza neuraminidase; derived from the crystal structure. (Adapted with permission from REF. 76 © (1997) American Chemical Society.)

potent inhibitor of the enzyme, and of the *in vitro* and *in vivo* replication of both influenza A and B virus[73]. Zanamivir was tailored to interact with conserved amino-acid residues within the active site of influenza A and B viral neuraminidase, and its inhibitory effect on the enzyme has proved to be predictive of the susceptibility of clinical isolates to the drug[74]. Meanwhile, zanamivir was shown to be efficacious and safe in the treatment (by inhalation) of influenza virus infections[75], and the drug has been licensed for clinical use.

Zanamivir has to be given topically (by inhalation), owing to its poor oral bioavailability. In attempts to identify potentially orally bioavailable inhibitors, a series of carbocyclic transition-state-based analogues were developed, in which the polar glycerol and guanidino groups of zanamivir were replaced by a lipophilic (3-pentyloxy) side chain and amino group, respectively, to give GS4071 (REF. 76). X-ray crystallographic studies showed that these groups of GS4071 could be accommodated within the active site of neuraminidase (FIG. 7). As aimed for, GS4071, when administered as the ethyl-ester prodrug (GS4104; oseltamivir; FIG. 2), is orally bioavailable and was found to be effective in protecting mice and ferrets against influenza infection[77]. Subsequently, oseltamivir was found to be effective and safe in the oral treatment and prevention of influenza virus infections[78,79], and has been licensed for clinical use.

Zanamivir and oseltamivir have paved the way for the development of similar structure-based neuraminidase inhibitors[80–82], such as the cyclopentane derivative RWJ270201, which have a comparable, or even better, efficacy profile in the mouse model of influenza. The clinical potential of RWJ270201 in the prevention and/or treatment of human influenza virus infections still needs to be established.

IMP dehydrogenase inhibitors

IMP dehydrogenase is a key enzyme in the *de novo* biosynthesis of purine mononucleotides: it is responsible for the NAD-dependent oxidation of IMP to xanthosine 5′-monophosphate (XMP), which is then further converted to GMP, GDP and GTP, and also from GDP, through dGDP, to dGTP. Inhibitors of IMP dehydrogenase might affect both RNA and DNA synthesis by reducing the intracellular pools of GTP and dGTP, respectively. Although IMP dehydrogenase is a cellular target, inhibitors targeted at this enzyme might inhibit viral RNA and/or DNA synthesis preferentially, as there is an increased need for such syntheses in virus-infected cells.

IMP dehydrogenase can be targeted by two types of inhibitor: competitive or uncompetitive with respect to the normal substrate, IMP. To the first category belongs ribavirin, which has been approved for clinical use as an aerosol for the treatment of RSV infections, and in combination with interferon-α for the treatment of HCV infections. To the second category belongs mycophenolic acid (FIG. 2), an immunosuppressing agent that has been approved, as its morpholinoethyl ester prodrug, for the prevention of acute allograft rejection following kidney transplantation. The X-ray crystal structure of IMP dehydrogenase, complexed with mycophenolic acid at the active site, has been reported at high resolution (2.6 Å)[83]. New congeners of both ribavirin (EICAR)[84] and mycophenolic acid (VX497)[85] have an activity spectrum as broad as ribavirin, but considerably greater potency. This activity spectrum encompasses both DNA and RNA viruses, including, among the latter, picorna-, toga-, flavi-, bunya-, arena-, reo-, rhabdo-, and, in particular, ortho- and paramyxoviruses.

Mycophenolic acid has marked activity against yellow fever virus and also markedly potentiates the inhibitory effects of the acyclic guanosine analogues acyclovir, penciclovir and ganciclovir against HSV, VZV and CMV infections, which could be of great clinical utility in organ-transplant recipients with these infections[86]. Furthermore, mycophenolic acid potentiates the activity of guanine-derived dideoxynucleoside analogues, such as abacavir, against HIV[87], which could be further exploited as a new combination strategy in the treatment of HIV infections.

Although IMP dehydrogenase inhibitors should, in their own right, be explored further for their potential in the treatment of various (+)RNA and (−)RNA viral infections, including haemorrhagic fever virus infections, current interest is mainly focused on their use in combination with (PEGYLATED) interferon-α in the treatment of HCV infections. This stems from the successful responses that have been seen following treatment of chronic hepatitis C with ribavirin in combination with interferon-α, in patients who did not respond to interferon alone[88].

Recently, ribavirin was shown to act as an RNA-virus mutagen, forcing RNA viruses into a lethal accumulation of errors, dubbed 'error catastrophe'[89,90]. The antiviral activity of ribavirin might, therefore, result from the lethal mutagenic effect following incorporation of ribavirin into the viral genome, and, obviously, this lethal mutagenesis might be enhanced by the inhibitory effect of ribavirin (in its 5′-monophosphate form) on IMP dehydrogenase and the consequent decrease in cellular

PEGYLATION
Addition of poly(ethylene glycol) (PEG) groups to proteins can increase their resistance to proteolytic degradation, improve their water solubility and reduce their antigenicity.

GTP pools (as mentioned above). The ability of ribavirin to force RNA viruses into error catastrophe has so far been shown only with poliovirus[89,90], and it remains to be verified whether the theory also holds for other RNA viruses, such as HCV, and other substrate analogues, such as EICAR.

S-adenosylhomocysteine hydrolase inhibitors

S-adenosylhomocysteine (SAH) hydrolase is a key enzyme in methylation reactions that depend on *S*-adenosylmethionine (SAM) as the methyl donor, including those methylation reactions that are required for the maturation of viral mRNA. In particular, (–)RNA viruses are crucially dependent on these methylations for the stability and functioning of their messenger RNA. SAH is both a product and an inhibitor of the methyltransferase reactions; however, SAH is rapidly hydrolysed by SAH hydrolase into homocysteine and adenosine, and this prevents the accumulation of SAH , which would otherwise lead to an inhibition of the SAM-dependent methylation reactions. Inhibitors of the SAH hydrolase could therefore lead to an accumulation of SAH, and consequent inhibition of the methylation reactions. Again, as noted for IMP dehydrogenase, SAH hydrolase is a cellular target, but as virus replication increases the need for such methylations, SAH hydrolase inhibitors might confer selective antiviral activity that could vary from one virus to another depending on their individual methylation needs.

Various adenosine analogues, such as carbocyclic adenosine, carbocyclic 3-deazaadenosine, neplanocin A, 3-deazaneplanocin A and their 5′-nor derivatives, have been described as potent inhibitors of SAH hydrolase[91]. All of these compounds have a characteristic antiviral activity spectrum, encompassing, in particular, poxviruses, (±)RNA viruses (reoviruses) and (–)RNA viruses (bunya-, arena-, rhabdo-, filo-, ortho- and paramyxoviruses). This includes several haemorrhagic fever viruses, such as Ebola haemorrhagic fever virus. In fact, a mouse model for Ebola haemorrhagic fever has been developed[92], and the SAH hydrolase inhibitors carbocyclic 3-deazaadenosine[93] and 3-deazaneplanocin A[94] were found to protect the animals against an otherwise lethal Ebola virus infection. So, SAH hydrolase inhibitors offer real potential for the treatment of haemorrhagic fever virus infections.

Conclusion

The strategies reviewed here for interfering with the key events in the viral replicative cycle have the potential to target virtually all important human viral pathogens. Several of these strategies, such as those aimed at viral DNA synthesis, viral polyprotein cleavage, and viral release from the cells (by means of the viral neuraminidase), have already provided a number of effective and useful therapeutics for the treatment of herpesvirus (HSV-1, HSV-2, VZV and CMV), retrovirus (HIV), hepadnavirus (HBV) and influenza virus infections. Further improvements along these lines could yield more efficacious and more selective antiviral compounds. This should by no means detract from other approaches, not addressed here, that might also be predicted to target viral compounds or virus-associated events. For example, agents that specifically bind to the picornaviral capsids (pleconaril), the HIV nucleocapsid, p7 (2,2′-dithiobisbenzamides), or the influenza virus A matrix (adamantanamine derivatives); glycosylation inhibitors (deoxynojirimycin derivatives); antisense oligonucleotides or ribozymes targeted at selected viral mRNAs; gene therapy approaches; immunotherapy; and so on. Whatever approach or strategy is followed to combat viral infections, the highest profit is likely to be obtained if two or more of these strategies are combined, especially in the treatment of chronic viral infections, such as HIV, HBV and HCV.

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Most virus infections are amenable to antiviral therapy, and this also holds for such feared viral pathogens as Ebola.

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New Antiviral Drugs that Target Herpesvirus Helicase Primase Enzymes

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KEY WORDS

HERPES SIMPLEX ▮ HSV ▮ HERPES DISEASE ▮ DRUG ▮ ANTIVIRAL AGENT ▮ INHIBITOR ▮ HELICASE PRIMASE ▮ BAY 57-1293 ▮ BILS 179 BS ▮ BILS 45 BS ▮ ACICLOVIR ▮ VALACICLOVIR ▮ PENCICLOVIR ▮ FAMCICLOVIR ▮ GANCICLOVIR ▮ VALGANCICLOVIR ▮ CIDOFOVIR ▮ FOSCARNET ▮ IDOXURIDINE ▮ TRIFLURIDINE ▮ BRIVUDINE ▮ FOMIVIRSEN ▮ TREATMENT

SUMMARY

Herpesviruses have infected the majority of the world's population and the associated diseases have plagued humanity since ancient times. Nine causative human herpesviruses have been identified so far. The first antiviral drug was launched in 1962, and since then several drugs for treating herpesvirus infections, which work via different mechanisms, have been developed. Current treatments abrogate or suppress disease symptoms but are not curative. A vaccine based on the OKA strain of varicella zoster virus is being marketed, but to date no therapeutic or prophylactic herpes vaccinations that can treat or stop spread of other herpes diseases are available. Herpes simplex virus causes mucocutaneous infections such as herpes genitalis (genital herpes) and herpes labialis (cold sores), the potentially sight-impairing herpetic eye disease, and life-threatening herpes encephalitis or disseminated disease. Recently, reports of helicase primase inhibitors, the first non-nucleosidic antiviral compounds, which are superior in pre-clinical profile to current herpes simplex virus medication, have been published. This review summarizes the data on helicase primase inhibitors and compares their pre-clinical profile with the established medical standard.

Introduction

OUR KNOWLEDGE OF the nine human herpesviruses, designated HHV-1 to HHV-8, has been compiled previously.[1] Based on the similarity of biological properties, the viruses were grouped into 3 subfamilies, namely neurotropic α-(HHV1 to 3) and lymphotropic β-(HHV5, 6A, 6B, 7) and γ-(HHV4 and HHV8) herpesviruses. Common names derive from clinical symptoms or historical reasons. For example, herpes simplex virus serotype 1 or 2 (HSV-1 or 2, the cause of herpes labialis and genitalis) is used for HHV-1 or 2, and varicella zoster virus (VZV, which causes chickenpox or zoster), Epstein–Barr virus, human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are synonyms for HHV-3, HHV-4, HHV-5 and HHV-8, respectively. The nomenclature of human herpesviruses, incidence and prevalence data, disease and symptoms, treatment options and genome details are summarized in Table 1.

Herpesviruses are omnipresent and thus herpes disease is ubiquitous. One key feature of herpesviruses is their ability to remain latent in their host after primary infection. They reactivate intermittently, from a pool of latently infected cells, on immune suppression and/or diverse internal and external stimuli such as physical or emotional stress, menses, corticosteroid use, fever, exposure to UV light and tissue damage. After primary infection therefore, the virus is not totally cleared by the immune system but persists chronically for life.

Options for therapy, both present and past,[1–3] and the significant challenge in establishing new treatments for herpes simplex disease have been reviewed recently.[4–5] Despite today's therapeutic standard, the key criteria in the search for novel therapeutics and treatment options include potency (including broad-spectrum activity); efficacy (especially upon delayed treatment); safety; growing resistance in immunocompromised patients; lack of therapeutic options for nucleoside-analogue resistant herpesviruses; the unsolved problem of latency and recurrences; and the mortality and morbidity associated with severe herpes disease. All these emphasize the medical need for new treatments.

The HHV genomes (Table 1; 125–230 kbp in size) have been sequenced and published.[6–8] They encode more than 50 genes essential for viral replication *in vitro* and/or *in vivo*. In principle, all essential viral molecules involved in viral replication are targets for chemotherapy. The most attractive gene targets have high sequence homology between different human herpesviruses and are involved in fundamental biochemical reactions such as viral DNA metabolism or viral proteases.[1] Antiviral targets involved in the replication of HSV DNA have been reported,[9–12] and new targets were discussed.[13] To replicate, many pathogens, including herpesviruses, encode their own helicase and/or primase to synthesize DNA.[1,5,13,14] The helicase separates or unwinds duplex viral DNA and the primase synthesizes RNA primers on the single-stranded DNA that serve as starting points for DNA synthesis performed by the viral DNA polymerase.[14–17] Inhibition of these initial or pre-requisite steps of DNA synthesis abrogates viral propagation and could lead to the development of potent drugs for treating and curing infection.[4–5]

Numerous approaches and strategies have been tested and considerable effort expended in the search for the next generation of anti-herpetic therapy (e.g. vaccination, immunotherapy and chemotherapy).[4] Despite this, it has been difficult to find a therapy that outperforms aciclovir (ACV), a drug discovered during the golden era of anti-metabolite research[11,12] (Nobel Prize in 1988).[18]

More recently, strategies such as structural determination by X-ray crystallography, electron microscopy, nuclear magnetic resonance, rational drug design, or approaches based on genomics, bioinformatics and target-based high-throughput screening (HTS), have been employed. The accumulation of new techniques is now beginning to reap rewards.

Drug Discovery and *In Vitro* Activity of Helicase Primase Inhibitors

Among the techniques used in drug discovery, turning natural metabolites into drugs (anti-metabolite research)

Table 1: Human herpesviruses: nomenclature, associated disease, licensed therapeutics and genome details

Virus	Virus Accession No; genome size; open reading frame (ORF)	Sub-family	Disease/symptoms	Treatment www.pdr.net	Prevalence [%]/ Incidence [number of cases]. Data from reference 1
HSV-1 Herpes simplex virus	HHV-1 X14112; 152 kb; ~84	α	Gingivostomatitis, herpes labialis, keratitis, encephalitis, neonatal herpes	ACV, VACV, PCV, FCV, (BVDU, PFA, TFT)	Children: ~20–40% Adults: ~50–90%
HSV-2 Herpes simplex virus	HHV-2 Z86099; 155 kb; ~84	α	Herpes genitalis, meningoencephalitis, neonatal herpes	ACV, VACV, PCV, FCV, (PFA)	5–50%, ~1.6 million cases/year (USA)
VZV Varicella zoster virus	HHV-3 X04370; 125 kb; ~69	α	Varicella (chickenpox), varicella zoster (herpes zoster)	ACV, VACV, PCV, FCV, BVDU, VZIG, BayGam®, Varivax®	Children: ~50–75% >85% by adulthood
EBV Epstein–Barr virus	HHV-4 V01555; 172 kb; ~128	γ	Mononucleosis (Glandular fever/ infectious mono-nucleosis), nasopha-ryngeal carcinoma		>80% by age 5 ~60–100%
HCMV Human cytomegalovirus	HHV-5 X17403; 229 kb; ~220	β	Immuno-compromised: pneumonia, retinitis, hepatitis, gastroenteritis. Congenital infection	GCV, VGCV, CDV, PFA, fomivirsen	Children: 10–30% Adults: ~60%, up to >90%
	HHV-6 A, B X83413, AF157706; 159–170 kb; 110 and 119	β	Febrile illnesses, exanthema subitum (roseola)		Infection in first 2 years of life. Approaching 100% in adulthood
	HHV-7 AF037218; 145–153 kb; 80	β	Febrile illnesses, exanthema subitum (roseola)		Infection in first 2 years of life. Approaching 100% in adulthood
KSHV Kaposi's sarcoma-associated herpesvirus	HHV-8 U75698 (UL); 134 kb; ~72	γ	Kaposi's sarcoma	Radiation, cytotoxic drugs, interferon-α	<3% up to 25%

Genomes: www.ebi.uk/genomes/index.html.
ACV, aciclovir; VACV, valaciclovir; PCV, penciclovir; FCV, famciclovir; BVDU, brivudine; GCV, ganciclovir; VGCV, valganciclovir; CDV, cidofovir; PFA, foscarnet; TFT, trifluridine; VZIG, varicella zoster immune globulin.

has been exceptionally successful and has produced ACV, valaciclovir (VACV), penciclovir (PCV), famciclovir (FCV), idoxuridine (IDU), brivudine (BVDU), vidarabine (Ara-A), trifluridine (TFT), ganciclovir (GCV), valganciclovir (VGCV), cidofovir (CDV) and foscarnet (PFA), all of which are marketed in many countries for the treatment of herpesvirus-caused disease.[1,3–5,9–12,19–27] Soon after publication of the HSV genome,[28] the enzymatic activity of a viral helicase primase,[17] and subsequently the first enzyme inhibitors, were reported *in vitro*.[5,29–32] More recently, potent inhibitors of HSV helicase primase have been discovered by random HTS of compound libraries.[33–40]

The lead compounds BILS 179 BS and BILS 45 BS were optimized from initial hits identified by using an enzyme-based HTS assay.[34] The development candidate BAY 57-1293 originated from a novel cell-based viral replication assay that mimics the smallest unit of infection.[36] A lead compound is the most active of a series of compounds related to a structure activity relationship (SAR) shown *in vivo*. The lead compound is the starting point for a strategic project, and during the project phase is optimized to a development candidate with potent *in vivo* activity, good pharmacokinetics and a safety profile suitable for clinical development.

Helicase primase was the pre-selected target in the case of BILS 179 BS and BILS 45 BS, but the mechanism of action of BAY 57-1293 was elucidated by selection of drug-resistant viruses and subsequent sequencing of a viral genome (HSV-1 F) and identification of resistance conferring genes by complementation analysis. Dose-dependent inhibition of the ATPase activity of purified helicase primase confirmed the target.

The broad spectrum anti-herpes activity of BAY 57-1293 against pseudorabies virus (PRV) and bovine herpes virus (BHV) and activity of thiazolyl-phenyl derivatives against HCMV have been reported.[36,38] Additional activities will probably be reported. The majority of comparable data for BILS 179 BS, BILS 45 BS and BAY 57-1293 relates to herpes simplex viruses,[34–40] thus the *in vitro* profile of the compounds against HSV-1 and HSV-2 is summarized and compared with the standard drugs in Table 2.

In contrast to the pre-prodrugs VACV and FCV (converted enzymatically *in vivo* to ACV and PCV, respectively[1,3,22–26]), and the prodrugs ACV and PCV, helicase primase inhibitors require no metabolic activation and directly inhibit their target. ACV and PCV are converted to ACV-monophosphate (MP) and PCV-MP, respectively, by viral thymidine kinase (TK) in infected cells. Subsequently, cellular kinases phosphorylate these monophosphorylated compounds to the active triphosphates (TP), which competitively inhibit binding of dGTP to the viral DNA polymerase and terminate chain elongation (ACV) or act as non-obligate terminators of chain elongation (PCV). The higher affinity of PCV for viral TK results in a longer half-life and higher intracellular concentrations of PCV-TP compared with ACV-TP. The apparent advantage of PCV is outweighed, however, by the lower inhibitory constants of ACV at the target level – the viral DNA polymerase. ACV is therefore slightly more potent in cell culture experiments than PCV. While the varying

Table 2. *In vitro* activity of helicase primase inhibitors against herpes simplex virus, compared with drugs used in the clinic[1–5,11,12,19–26]

	Drugs and investigational compounds					
***In vitro* (target)**	**Aciclovir (ACV)**	**Valaciclovir (VACV), prodrug of ACV**	**Penciclovir (PCV)**	**Famciclovir (FCV), prodrug of PCV**	**BAY 57-1293**	**BILS 179 BS**
Thymidine kinase (TK) inhibition constant (Ki) (μM)	172	See ACV	1.5	See PCV		
Polymerase (Pol) Ki (μM)	0.07–0.08	See ACV	8.5	See PCV		
Helicase IC_{50} (μM)						1.3
Primase IC_{50} (μM)						0.15
ATPase IC_{50} (μM)					0.03	0.43
Cell culture EC_{50} (HSV; μM)	0.5–1.0	See ACV	2–4	See PCV	0.01–0.02	0.027–0.1
Correlation of EC_{50} with viral load	Strong	See ACV	Strong	See PCV	Weak	
Selectivity index (SI)	~250	See ACV	~200	See PCV	>2000	>2000
Half-life (h) in cells	0.7–1.0 ACV-triphosphate	See ACV	~10 PCV-triphosphate	See PCV		
Genes containing mutations conferring resistance	*TK/Pol*	*TK/Pol*	*TK/Pol*	*TK/Pol*	U_L5/U_L52	U_L5
Frequency of drug-resistant viruses	~$1x10^{-3}$ (range $1x10^{-2}$–$1x10^{-5}$)	See ACV	~$1x10^{-3}$ (range $1x10^{-2}$–$1x10^{-5}$)	See PCV	~0.5–$4x10^{-6}$	~0.5–$4x10^{-6}$
Pharmacokinetic profile						
$T_{1/2}$ (h) Plasma half-life of drug in species	2–3 man	~2.9 ACV man	~2.0 man	2.0 PCV man	6 mice	0.9 mice
T_{max} (h) Time to reach C_{max} of drug	1.5–2.5 man	0.5–1.0 VACV		0.5–1.0 FCV	2.0–3.0	0.4–0.5
Peak concentration C_{max}	200 mg tablet ACV	500 mg tablet VACV	See FCV	125 mg tablet FCV		
Dose (mg/kg) orally	2.7 man	6.7 man		1.7 man	1 mice	25 mice
C_{max} (mg/l)	0.58	3.28 ACV		0.8 PCV	1.8	14.4
C_{max} (μM)	2.6	14.6 ACV		3.2 PCV	4.4	31.5
Bioavailability	10–25%	54%	<5%	77%	>60%	~50%
Protein binding	9–33%	See ACV	<20%	see PCV	>95%	>95%

IC_{50}, 50% inhibitory concentration; EC_{50}, effective concentration inhibiting viral growth by 50%.

inhibitory constants at the target level shown in Table 2 are of the same order of magnitude for all compounds, the helicase primase compounds are up to (or nearly) two orders of magnitude more potent than the listed nucleosidic drugs in inhibiting viral replication in infected cell cultures. The IC_{50} of the thiazolylamide compounds is less dependent on viral load and the lower IC_{50} values correlate with a higher selectivity index. The novel compounds are active against clinical isolates and due to a different mechanism of action also block viral replication of nucleoside-, nucleotide- and pyrophosphate analogue-resistant HSV, irrespective of the permissive cell line being infected and the compound being protein bound. Furthermore, combination therapy using BAY 57-1293 and nucleosidic drugs shows a synergistic effect *in vitro*.[41]

The frequency at which drug-resistant viruses can be selected in the presence of compound *in vitro* is an order of magnitude lower in helicase primase inhibitors compared with nucleosidic drugs (Table 2).[36,42] So far, mutations conferring resistance to helicase primase inhibitors have been identified in the U_L5 gene of HSV. In addition, a minority of thiazolylamide-resistant viruses have mutations in the U_L52 gene. The thiazolyl-phenyl compounds (e.g. BILS 179 BS) are not cross-resistant to this thiazolylamide-resistant virus (U_L52 A897T), leading to the conclusion that the helicase primase inhibitors bind to the same site on the helicase enzyme (encoded by the U_L5 gene) but that thiazolylamides also bind the primase (U_L52 gene product). The fact that pharmacophores of BAY 57-1293 and BILS 179 BS can be fused to an antiherpes compound supports these findings.[5] A pharmacophore is a chemical group or moiety which binds to the target. Compounds with matching pharmacophores have similar physical or biomedical properties and thus a common SAR. Finally, no comparable data exist regarding development of resistance *in vivo*.

At this stage, members of the thiazolylamide series, e.g. BAY 57-1293, are more potent than the respective compound of the thiazolyl-phenyl series, BILS 179 BS, *in vitro* and *in vivo*. Projects focusing on the aminothiazolyl-phenyl compound class were, however, discontinued before the lead compounds could be optimized to a development candidate.

In Vivo Activity of Helicase Primase Inhibitors in Animal Models

In contrast to the non-optimized screening hits (a compound with statistically significant activity in a test system or HTS assay) T-157602[33,39] and ER622,[32] profound anti-HSV activity has been published for the

the development candidate BAY 57-1293.[36,37,40] The potency and efficacy of the orally bioavailable helicase primase inhibitors in diverse animal models are compared in Table 3.

BILS 179 BS and BILS 45 BS are nearly equipotent to ACV in the murine lethal challenge model (ED_{50}=24/35 and 20/38 versus 22/16 mg/kg, HSV-1/HSV-2, three times a day, orally). The lower potency against HSV-2 correlates with a less pronounced efficacy in the murine model of intravaginal HSV-2 infection.[34] The efficacy of BILS 179 BS is significantly superior to ACV in mouse models of cutaneous HSV-1 disease at equivalent doses (three times daily as well as once daily), especially when treatment is delayed by 65 h post infection.[34] It appears that the lower IC_{50} values for BILS 179 BS compensate for deficits in the pharmacokinetic profile. Overall, for a lead compound the efficacy in diverse animal models is remarkable.

After peroral administration, BAY 57-1293 is at least 20 times more potent than VACV ($ED_{50\ HSV\text{-}2}$ 0.5 versus 15 mg/kg VACV [three times daily]), even when applied once daily.[36,37,40] Apart from the murine lethal challenge model, selected drugs[36] show superior dose-dependent efficacy against HSV when compared with launched nucleosidic drugs in the rat lethal challenge,[37] murine zosteriform spread[36,37] and guinea pig models.[36] In the zosteriform spread model, which mimics more closely the clinical situation of cutaneous infections, superior efficacy has been demonstrated, especially with delayed treatment and even after onset of disease.[36,37] In particular, when treatment with BAY 57-1293 was stopped, no rebound of disease was observed in this model. In contrast to VACV, BAY 57-1293 continued to show efficacy and successfully suppressed herpes spread while animals treated with 60 mg/kg VACV eventually died.[36,37] In addition, BAY 57-1293 was more effective than ACV when administered topically in an alcoholic formulation, or when ocular herpes infections were treated in a murine keratitis model using topical eye drop formulations (2% wt/vol).[37]

The guinea pig model of intravaginal HSV-2 infection resembles the clinical situation of genital herpes in humans. BAY 57-1293 was superior to VACV in this recurrent disease was measured in terms of a disease score.[36] BAY 57-1293 not only completely suppressed clinical symptoms of primary disease but also almost totally abrogated viral shedding.[36] Treatment of acute disease also decreased the occurrence of recurrent vesicles in the follow-up period. Initiation of therapy after appearance of vesicles showed that BAY 57-1293 is clearly more efficacious than VACV in reducing time to healing. Treatment of guinea pigs after healing of untreated primary disease reduced the frequency of recurrences and their severity within the treatment interval.

In summary, BAY 57-1293 is the most potent and efficacious drug candidate in all animal models tested.

Pharmacokinetics and Safety Profile

The pharmacokinetic profile of BAY 57-1293 can be described as a high exposure, low clearance, long plasma half-life, high oral bioavailability drug with no evidence of a drug interaction potential.[36] An outstanding feature, compared with the other drugs or lead compounds in Table 2, is its long plasma half-life ($T_{1/2}$) of more than 6 h in mice, rats and dogs. The solubility of most of the helicase primase inhibitor derivatives is low and protein binding is high compared with nucleosidic drugs. The resulting small free fraction correlates with a high partition coefficient.

Exploratory toxicology and safety pharmacology studies did not reveal any relevant safety findings at 30 mg/kg, 100 mg/kg and 300 mg/kg Bay 57-1293 (once daily orally) in a 4-week chronic toxicity study in rats and dogs.[36] However, administration to rats of more than 50 times the ED_{50} dose leads to diet and species-dependent side-effects based on inhibition of carbonic anhydrase enzymes in rodents.

The structural formula of BAY 57-1293 resembles the structure of acetazolamide, a diuretic drug known to inhibit carbonic anhydrase (CA) enzymes.[43] The structural formulae are shown in Figure 1 for comparison. The compounds share a primary sulphonamide group bound to aromatic heterocyclic ring systems, the common pharmacophore of the CA inhibitor class of diuretic

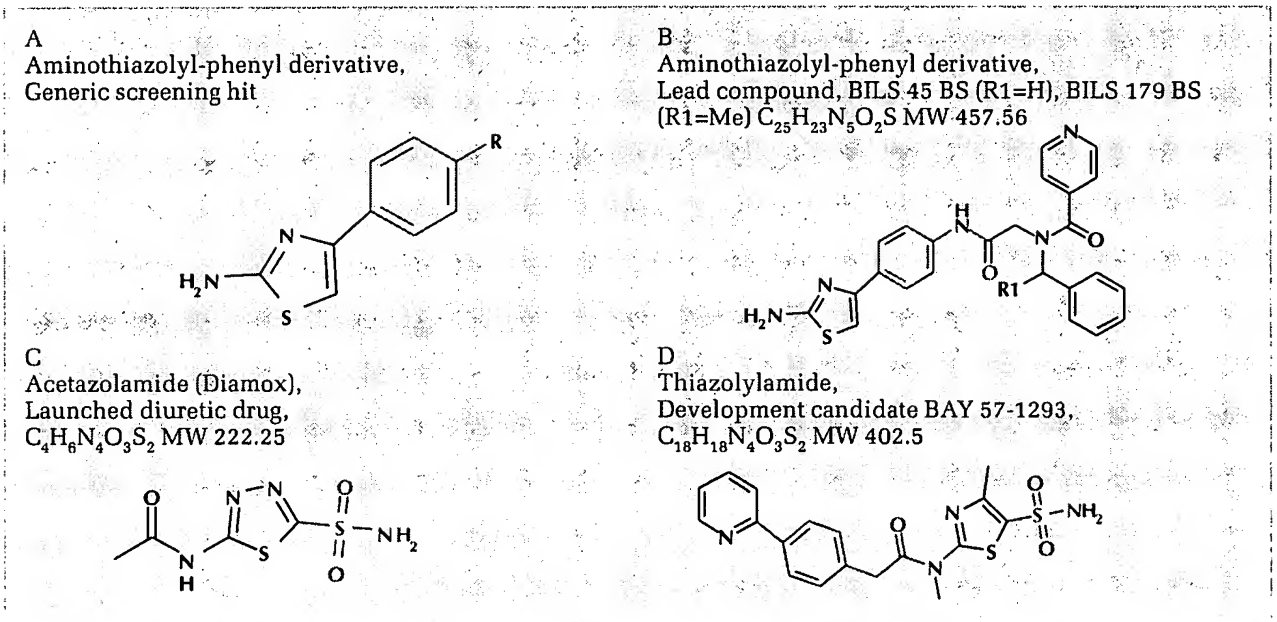

Figure 1:
Structural formulae of the helicase primase inhibitors and acetazolamides. A) The generic compound discovered by various screening test systems: Boehringer/Biomega;[34,38] Merck (ER622, R=Br);[32] Tularik (T-157602, R=Cl);[33,39] and Bayer (R=H).[5] B) The optimized compounds with in vivo *antiviral activity (Boehringer/Biomega)[34,35,38] and D) the anti HSV development candidate BAY 57-1293.[36,37,40] The pharmacophores of acetazolamide (C) and BAY 57-1293[36] (D) binding to carbonic anhydrase enzymes via the primary sulphonamide linked to an aromatic heterocycle. Diamox, N-(5-Sulphamoyl-[1,3,4] thiadiazol-2-yl)-acetamide (C), and BAY 57-1293, N-[5-(aminosulphonyl)-4-methyl-1,3-thiazol-2-yl]-N-methyl-2-[X]-acetamide (D) are shown for comparison.*

Table 3. Potency and efficacy of helicase primase inhibitors in diverse animal models compared with drugs used in the clinic[1–5,11,12,19–26]

Mouse lethal challenge model (HSV-1 walki, HSV-2 MS, three times daily treatment)[34–37]

	Aciclovir (ACV)	Valaciclovir (VACV)	Penciclovir (PCV)	Famciclovir (FCV)	Ganciclovir (GCV)	BAY 57-1293	BILS 179 BS	BILS 45 BS
ED_{50} (mg/kg) HSV-1/ HSV-2	22/16	17/15	See FCV	17/24	2.5/1.0	0.5/0.5	24/35	20/38

Mouse lethal challenge model (HSV-2 MS, once daily treatment)[37]

	VACV	BAY 57-1293
ED_{50} (mg/kg)	~120	~4

Effect on viral burden in HSV-1 (walki) infected mice after cessation of therapy[36,37]

	Recurrence	No recurrence

Murine zosteriform spread model (three times daily). Delayed oral treatment[36,37]

(mg/kg)	60 and 240	15 and 60
HSV-2	Rebound of infection	No rebound of infection

Cutaneous HSV-1 KOS infection of hairless (SKH-1) mice[34]

	ACV	BILS 179 BS
Treatment three times daily orally		
ED_{50} (mg/kg per day)	25	31
Treatment (3 h p.i., 5 days) once daily orally		
Reduced pathology (AUC)	~40% (50 mg/kg); ~45% (100 mg/kg)	83% (50 mg/kg); 97% (100 mg/kg)
Delayed treatment (three times daily, 65 h p.i.)		
(AUC)	Weak effect Weak effect	49% (100 mg/kg per day) 75% (200 mg/kg per day)

Topical treatment of murine mucocutaneous and ocular HSV-2 infection[37] (2% wt/vol, twice daily for cutaneous and five times daily for ocular herpes). Percentage of animals showing signs of infection on Day 10

	ACV	BAY 57-1293
Mucocutaneous	~50	0
Ocular herpes	~30	0

Rat lethal challenge model (three times daily)[37]

	VACV	BAY 57-1293
ED_{50} (mg/kg)	~75–125	~0.5

Mouse model of intravaginal HSV-2 HG52 infection.[34] Reduction of disease correlates with reduced viral titres

	ACV	BILS 179 BS
Treatment (3 h p.i., four times daily, for 7 days)		
Disease ED_{50}	~150 (mg/kg per day)	46 (mg/kg per day)
Mortality ED_{50}	~150 (mg/kg per day)	33 (mg/kg per day)

Guinea pig model of intravaginal HSV-2 strain MS infection[36]

	VACV	BAY 57-1293
Acute treatment (twice daily)	Disease symptoms (150 mg/kg)	No disease symptoms (20 mg/kg)
Reduction of recurrences after acute treatment	Not statistically significant Three times daily (100 mg/kg)	Almost completely Twice daily (30 mg/kg)
Delayed treatment efficacy	Twice daily (150 mg/kg) Progression of disease symptoms Minor reduction (2 days) time to healing (11 days versus 9 days)	Twice daily (20 mg/kg) Abrogates progression of disease symptoms. Major reduction (7 days) time to healing (11 days versus 4 days)
Suppression of primary disease	Weak effect (Twice daily [150mg/kg])	Profound effect No symptoms (twice daily [20 mg/kg])
Suppression of recurrent disease	Yes; during treatment interval (Twice daily [150 mg/kg])	Yes; during treatment interval (Twice daily [30 mg/kg])
Suppression of viral shedding	Weak effect (same order of magnitude compared with placebo)	Almost totally abrogated. Log 0.6 ± 0.6 BAY 57-1293 versus Log 3.7 ± 0.8 Placebo (PFU/ml)

EC_{50}, effective concentration inhibiting viral growth by 50%; h p.i., hours post infection; PFU, plaque forming units.
ED_{50}, equivalent dose – the dose required to produce a specific effect in 50% of the test population.
AUC, area under curve.

leads to an increase in urine pH and sodium ion concentrations, due to secondary inhibition of the sodium/proton antiporter, and is a prerequisite for the hyperplasia observed.[44] The weak diuretic effect observed when overdosing animals with more than 50 times the antiviral ED_{50} of BAY 57-1293 leads to reversible, cell-specific, diet dependent, transient hyperplasia in urethra and bladder epithelia of rodents only, but not in other species including humans.[44–48] This known effect, caused specifically by CA inhibitors, has been demonstrated in a series of articles.[45–48] Importantly, diet alone can completely prevent development of this reversible, transient species-specific hyperplasia, even at 600 times the antiviral ED_{50} (~ 0.5 mg/kg) established in the murine and rat lethal challenge models.

The high unspecific binding to protein (>95%) and the specific binding to CA (iso) enzymes result in a low free fraction of the drug *in vivo*, and distribution into at least five fractions at the target level, namely: unbound, membrane/lipid-, protein-, CA- and helicase primase bound. The binding affinity is highest for the helicase primase guaranteeing inhibition of the binding site on the target, and the low free fraction reduces the probability of side-effects. Overall, BAY 57-1293 is remarkably safe and non-toxic in animals.

Conclusions

Chemotherapy for herpes diseases with nucleoside (ACV, VACV, PCV, FCV, GCV, VGCV, TFT, IDU, BVDU) or nucleotide (CDV) analogues and pyrophosphate mimetics (PFA) is well established,[1–3] but does not completely satisfy the medical need.[3–5] Diseases caused by neurotropic α-herpesviruses (HSV, VZV) are predominately treated by systemic application of ACV, VACV, FCV and less often with BVDU or topical formulations of ACV and PCV, whereas GCV, VGCV and CDV were launched to treat HCMV infections. Due to its superior safety profile, ACV replaced systemic Ara-A soon after its launch in the early 1980s. Topical treatment of herpetic eye disease with IDU was replaced by USA) or ointments of ACV and/or TFT elsewhere. CDV and PFA are licensed for CMV retinitis in AIDS patients and PFA is also used as second line therapy (iv and topical) for mucocutaneous ACV-resistant HSV infections. Fomivirsen is an antisense therapeutic for intravitreal injection. For disease treatment and structural formulae, see references 1–3, 27 and 3–5, respectively.

The nucleoside or nucleotide analogues are obligate or non-obligate chain terminators of DNA-polymerization, making them potentially mutagenic. This is well documented for GCV, IDU, TFT, Ara-A and CDV.[27]

In summary, the established therapies can reduce, abrogate or suppress herpes disease symptoms, but do not cure the disease or reduce frequency of recurrent outbreaks after discontinuing treatment. Therapy with ACV, VACV and FCV is effective and safe, but the poor safety profiles of GCV, VGCV, CDV and PFA limit prescriptions to carefully evaluated cases. Overall, these drugs have broad anti-herpes activity (Table 1) but only moderate efficacy, especially when initiation of treatment is delayed.

In contrast to the pre-prodrugs VACV, FCV and VGCV or the prodrugs ACV, PCV and GCV, the new class of helicase primase inhibitors requires no activation to inhibit the viral target, and is the most active against HSV to date. Furthermore, their different mechanism of action and the observed synergy of BAY 57-1293 and DNA-polymerase inhibitors[41] *in vitro* are important when considering combination therapy. The antiviral spectrum of BAY 57-1293 includes PRV and BHV, whereas the aminothiazolyl-phenyl compound class has weak activity against HCMV. Other activities may be reported in the future. The aminothiazolyl-phenyl compounds (e.g. BILS 179 BS) have not been optimized to a development candidate, whereas BAY 57-1293 is suitable for clinical development. The good oral bioavailability and long plasma half-life allows for once-daily dosing, and this factor, together with its potency *in vitro*, makes it the most active compound in diverse animal models.

Table 4 compares relevant parameters of a potential product profile. The potency and efficacy of BAY 57-1293 compared with VACV, even using a once-daily regimen and especially when treatment is delayed, are

Table 4: Product profiles of the nucleosidic drug valaciclovir,[19,20,22–24] targeting viral thymidine kinase (TK)/DNA polymerase, compared with BAY 57-1293, a member of the novel class of helicase primase inhibitors,[36,37,40] in diverse animal models of HSV infection. For details see Table 2 and Table 3

	Valaciclovir	BAY 57-1293
Potency/efficacy	Moderate	Superior
Frequency of resistant viruses		
in vitro	High	Low
in vivo	Low	No data available
Cross resistance in class	Yes	Yes (exception U_L52 viral mutants)
Cross resistance between classes	No	No
Once-daily treatment	Yes	Yes (superior)
Delayed treatment	Difficult to demonstrate significant activity	Profound activity
Rebound of disease after cessation of treatment	Yes	No (reduced)
Time to healing	Significant reduction only when treatment initiated within 48 h after onset of disease	Significant reduction
Reduction of morbidity and mortality of herpes encephalitis or disseminated disease	Yes	Yes (superior)
Viral shedding*	Reduction	Nearly complete suppression
Suppression of disease	Yes	Yes (superior)
Reduction of recurrences	No	Yes (first data in animal models after treatment of primary disease)

*Correlates with better IC_{50} (IC_{50}, 50% inhibitory concentration) as a function of increasing viral load.

mortality and morbidity of severe herpes disease (e.g. herpes encephalitis or disseminated disease), significant shortening of time to healing compared with established therapy, and a decreased likelihood of direct rebound of disease after cessation of treatment. Almost complete inhibition of viral shedding results in effective suppression therapy, raising the hope of reducing viral spread. The effective inhibition of viral propagation *in vivo* correlates with a superior IC_{50} as a function of increasing viral load (m.o.i.) *in vitro*, a situation observed for the smallest unit of infection and during development of disease symptoms *in vivo*.

The possibility of reduced recurrences after therapy with BAY 57-1293 for primary HSV disease remains to be elucidated in the clinic, but overall this class of drugs has significant potential for treating HSV disease, including strains resistant to current therapeutics.

Dr Kleymann is a former employee of Bayer Pharmaceuticals, Wüppertal, Germany, and the applicant of the patent covering the helicase primase inhibitors in selected countries. He has recently habilitated at the University of Tübingen, Faculty of Chemistry and Pharmacy, as a prerequisite to taking a professorship in Germany.

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ANTIVIRALS AND ANTIVIRAL STRATEGIES

Erik De Clercq

Abstract | In recent years, the demand for new antiviral strategies has increased markedly. There are many contributing factors to this increased demand, including the ever-increasing prevalence of chronic viral infections such as HIV and hepatitis B and C, and the emergence of new viruses such as the SARS coronavirus. The potential danger of haemorrhagic fever viruses and eradicated viruses such as variola virus being used as bioterrorist weapons has also increased the profile of antiviral drug discovery. Here, the virus infections for which antiviral therapy is needed and the compounds that are available, or are being developed, for the treatment of these infections are described.

Interest in the development of new antiviral compounds is mainly fuelled by two considerations: what is the requirement for a specific antiviral drug against the virus infection concerned (and, linked to this, could it reasonably be expected that the virus infection would be controlled by using the antiviral drug); and which antiviral drugs are available to treat or prevent the virus infection concerned (or which antiviral strategies could be pursued to meet the demand). The purpose of this article is to examine how different virus infections should be approached from a therapeutic viewpoint — not only those virus infections that we are familiar with, but also new or old virus infections that could emerge or re-emerge, respectively. The basic strategies that are used to design antiviral drugs have been described previously[1]. Here, I will evaluate their usefulness, or potential usefulness, in the control of virus infections. The antiviral drugs that have been formally licensed for medical use are listed in BOX 1. The chemical structures of some of the compounds discussed in this article are shown in FIG. 1; the remaining compounds that are discussed are shown in online supplementary information S1 (figure). The viral and/or cellular targets for antiviral agents and potential antiviral agents are presented in TABLE 1.

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Parvovirus infections

The only parvovirus that is pathogenic for humans is B19, which is responsible for so-called fifth disease, or erythema infectiosum, in children. Although complications such as arthritis, aplastic crisis (reticulocytopoenia), myocarditis and hydrops fetalis (during pregnancy) can occur after infection with B19 virus, no serious attempts have been made to develop either preventative or therapeutic measures for B19-virus-associated disease and the question of whether any efforts should be made to develop a vaccine or cure for this disease remains open to debate.

Polyomavirus infections

The polyomaviruses JC and BK viruses have been associated with, and are thought to be responsible for, progressive multifocal leukoencephalopathy (PML) and haemorrhagic cystitis, respectively, in patients with AIDS. Several anecdotal case reports have indicated that cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC)] is effective in the treatment of PML in AIDS patients[2] if it is given at the dosage recommended for the treatment of human cytomegalovirus (HCMV) retinitis in AIDS patients — intravenously, 5 mg kg^{-1} $week^{-1}$ for 2 weeks then 5 mg kg^{-1} every 2 weeks, with concomitant probenecid administration. The activity of cidofovir against both primate and murine polyomaviruses has been demonstrated in cell culture *in vitro*[3]. So far, no other antiviral drugs have been proven to be effective against polyomavirus infections.

Box 1 | **Approved antiviral drugs**

HIV infections

Nucleoside reverse transcriptase inhibitors (NRTIs):

Zidovudine: 3′-azido-2′,3′-dideoxythymidine (AZT) | Didanosine: 2′,3′-dideoxyinosine (ddI) | Zalcitabine: 2′,3′-dideoxycytidine (ddC) | Stavudine: 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) | Lamivudine: (–)-β-L-3′-thia-2′,3′-dideoxycytidine (3TC) | Abacavir (ABC): 2-amino-6-cyclopropylaminopurin-9-yl-2-cyclopentene | Emtricitabine: (–)-β-L-3′-thia-2′,3′-dideoxy-5-fluorocytidine ((–)-FTC)

Nucleotide reverse transcriptase inhibitors (NtRTIs):

Tenofovir disoproxil: bis(isopropoxycarbonyloxymethyl)ester of (*R*)-9-(2-phosphonylmethoxypropyl)adenine

Non-nucleoside reverse transcriptase inhibitors (NNRTIs):

Nevirapine | Delavirdine | Efavirenz

Protease inhibitors (PIs):

Saquinavir | Ritonavir | Indinavir | Nelfinavir | Amprenavir | Lopinavir | Atazanavir

Fusion inhibitors (FIs):

Enfuvirtide : Pentafuside (T-20)

HBV infections

Lamivudine | Adefovir dipivoxil: bis(pivaloyloxymethyl)ester of 9-(2-phosphonylmethoxyethyl)adenine

HSV and VZV infections

Acyclovir and its oral prodrug valaciclovir | Penciclovir and its oral prodrug famciclovir | Idoxuridine: 5-iodo-2′-deoxyuridine (IDU) | Trifluridine: 5-trifluoro-2′-deoxythymidine (TFT) | Brivudin: (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU)

CMV infections

Ganciclovir and its oral prodrug valganciclovir | Foscarnet: phosphonoformic acid (PFA) trisodium salt | Cidofovir: (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) | Fomivirsen: antisense (phosphorothioate) oligonucleotide

Influenza virus infections

Amantadine | Rimantadine | Zanamivir | Oseltamivir

HCV infections

(Pegylated) IFN-α | Ribavirin

Papillomavirus infections

There are several clinical manifestations of human papillomavirus (HPV) infection, which include verruca vulgaris, plantar warts, hypopharyngeal, oesophageal, laryngeal and respiratory papillomatosis, genital warts (condylomata acuminata), cervical intraepithelial neoplasia (CIN) (which can develop into cervical carcinoma), vulvar intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN) and perianal intraepithelial neoplasia (PAIN). When injected intralesionally or applied topically as a 1% gel or cream, cidofovir has proved highly efficacious in causing regression of many HPV-associated lesions (including laryngeal papillomas and anogenital warts) with no or few recurrences. Any recurrences have responded promptly to another course of cidofovir therapy[2]. The inhibitory effect of cidofovir on the proliferation of HPV-infected cells could be attributed to the induction of apoptosis in these cells[4] and there is evidence that cidofovir restores p53 function in HPV-associated cancers[5].

Two other antiviral agents have specificity for HPV infections — the acyclic nucleoside phosphonate analogues PMEG [9-(2-phosphonylmethoxyethyl)guanine] and cPr-PMEDAP [9-(2-phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine], which selectively inhibit HPV-16-positive cells in organotypic co-cultures of primary normal human keratinocytes with cervical carcinoma cells[6].

Adenovirus infections

Adenovirus infections in immunocompetent individuals are generally self-limiting, and neither preventative nor therapeutic measures (vaccination or antiviral therapy) are used. However, in allogeneic haematopoietic stem-cell transplant (HSCT) recipients, adenovirus infections can be severe. In these patients, according to anecdotal reports, cidofovir has been shown to be to be effective in suppressing adenovirus infection, whereas ribavirin and vidarabine have not[7]. At present, cidofovir seems to be the only antiviral drug that could be successfully used to treat adenovirus infections, particularly in HSCT recipients[2].

α-herpesvirus infections

The α-herpesviruses include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella–zoster virus (VZV). They can cause both primary infections (for example, HSV-1 causes gingivostomatitis, encephalitis and eczema herpeticum, HSV-2 causes genital and neonatal herpes, and VZV causes varicella (chicken-pox)) and recurrent infections (for example, HSV-1 causes herpes labialis and herpetic keratitis, HSV-2 causes genital herpes and VZV causes herpes zoster). These viruses can also cause severe, disseminated or progressive mucocutaneous infections in immunosuppressed patients.

Adequate treatments that are available for α-herpesvirus infections[8] include: acyclovir and its oral prodrug

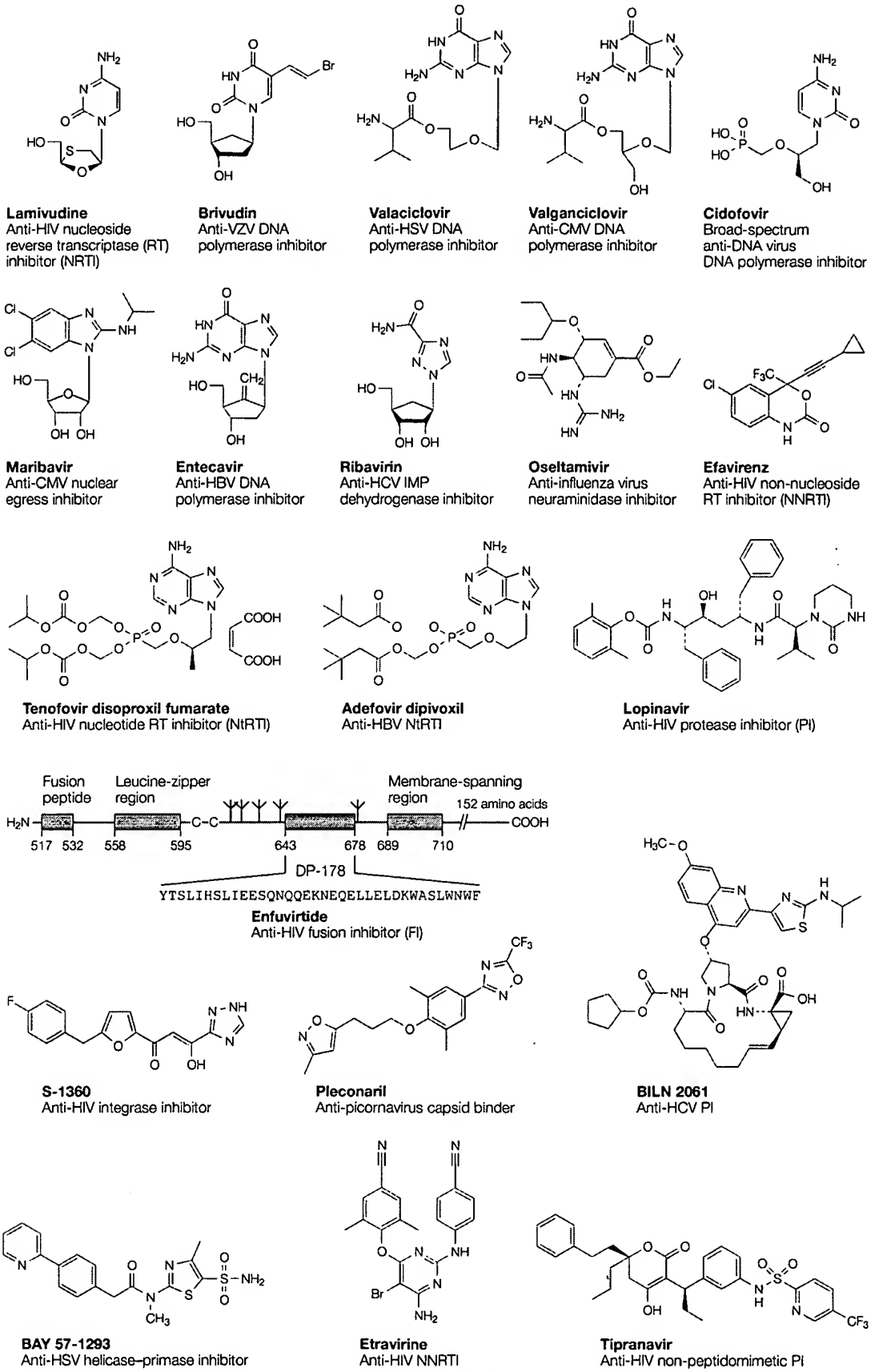

Figure 1 | **Structures of selected licensed antiviral drugs and compounds still in clinical or preclinical development.** For further detail, see REF. 8. For structures of the other compounds discussed in this article, see online supplementary information S1 (figure).

Table 1 | **Viral and cellular targets for antiviral agents**

Virus	Viral target	Cellular target	Comments
Parvovirus	DNA polymerase	–	Remains to be explored
Polyomavirus	DNA polymerase	–	Remains to be explored
Papillomavirus	DNA polymerase, E6, E7	–	Remains to be explored
Adenovirus	DNA polymerase	Cell-associated factors	Remains to be explored
α-herpesvirus (HSV-1, HSV-2, VZV)	Thymidine kinase DNA polymerase Helicase–primase	–	Target for activation Target for inhibition Target for inhibition
β-herpesvirus (HCMV, HHV-6, HHV-7)	Protein kinase DNA polymerase Terminase	–	Target for activation Target for inhibition Target for inhibition
γ-herpesvirus (EBV, HHV-8)	DNA polymerase	–	Target for inhibition
Poxvirus (for example, variola and vaccinia viruses)	DNA and RNA polymerases	Several*	Remains to be explored
Hepadnavirus (HBV)	DNA polymerase (Reverse transcriptase)	–	Target for inhibition
Picornavirus (Enteroviruses and Rhinoviruses)	Viral capsid RNA polymerase	–	Target for inhibition Remains to be explored
Flavivirus (for example, yellow fever and dengue viruses)	RNA polymerase	–	Remains to be explored
Arenavirus (for example, Lassa)	RNA polymerase	Several*	Remains to be explored
Bunyavirus (for example, Crimean–Congo)	RNA polymerase	Several*	Remains to be explored
Togavirus (for example, Western Equine encephalitis virus)	RNA polymerase	Several*	Remains to be explored
Rhabdovirus (Rabies virus)	RNA polymerase	Several*	Remains to be explored
Filovirus (for example, Ebola virus)	RNA polymerase	Several*	Remains to be explored
Hepacivirus (HCV)	RNA polymerase, RNA helicase, Viral protease	–	Being investigated
Orthomyxovirus (Influenza)	Matrix (M2) protein Neuraminidase	–	Target for inhibition Target for inhibition
Paramyxovirus (RSV)	Fusion polypeptide	Several*	Being explored
Coronavirus (SARS-CoV)	Several‡	–	Being explored
Reovirus (Rotavirus)	–	Several*	Remains to be explored
Retrovirus (HIV)	Several§‖	Several¶	Established or being explored

*Inosine 5′-monophosphate (IMP) dehydrogenase, S-adenosylhomocysteine (SAH) hydrolase, oritidine 5′-phosphate (OMP) decarboxylase and cytosine 5′-triphosphate (CTP) synthetase. ‡Spike (S) protein, RNA polymerase (replicase), RNA helicase and viral protease. §Fusion polypeptide (viral glycoprotein gp41), reverse transcriptase and viral protease. ‖Viral glycoprotein gp120, integrase and transcription transactivator (TAT). ¶Integration- and transcription-associated factors.

valaciclovir; penciclovir and its oral prodrug famciclovir; and brivudin (BVDU). BVDU has now been licensed in several European countries for the treatment of herpes zoster. Acyclovir and penciclovir are acyclic nucleoside analogues; in addition, some carbocyclic guanosine analogues (such as A-5021 and cyclohexenylguanine) and methylenecyclopropane analogues of nucleosides (such as synguanol), have been accredited with potent activity against HSV-1, HSV-2 and VZV[9]. Some of the methylenecyclopropane analogues have also proved effective against β- and γ-herpesviruses and hepatitis B virus (HBV)[10,11].

As acyclic nucleoside analogues require phosphorylation by the virus-encoded thymidine kinase (TK) to exert their antiviral activity (FIG. 2), they do not inhibit the TK-deficient HSV or VZV strains that can occasionally arise, particularly in immunocompromised hosts. In this situation, infections should be treated with foscarnet, a pyrophosphate analogue, or the acyclic nucleoside phosphonate cidofovir, neither of which depend on the HSV or VZV TK for their antiviral action (FIG. 3).

Also dependent on the activity of a viral TK, but specifically the VZV-encoded TK, are the bicyclic furo(2,3-d)pyrimidine nucleoside analogues (BCNAs) Cf 1368, Cf 1369, Cf 1743 and Cf 1742. These compounds inhibit VZV replication at subnanomolar concentrations and, even at 100,000-fold greater concentrations, are not toxic to host cells[12,13]. Unlike the 'classical' anti-VZV compounds, the BCNAs are selective for VZV and are therefore inactive against HSV-1 and HSV-2. The alkyl(phenyl) side chain on the furanyl ring of the BCNAs is an important determinant of the specificity of these compounds[14].

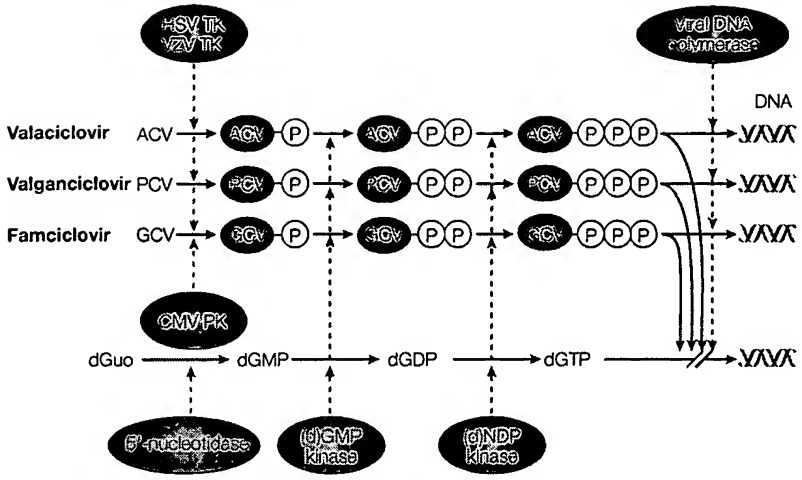

Figure 2 | **The mechanism of action of acyclic nucleoside analogues.** Examples of these compounds include acyclovir and its oral prodrug valaciclovir, penciclovir and its oral prodrug famciclovir, and ganciclovir and its oral prodrug valganciclovir. These acyclic nucleoside analogues require phosphorylation by the viral thymidine kinase (TK) to exert their antiviral activity for HSV and VZV (valaciclovir and famciclovir) and a protein kinase (PK; UL97) for CMV (valganciclovir).

New anti-HSV agents that target the viral helicase–primase complex[15] — the thiazolylphenyl derivatives BILS 179BS and BAY 57-1293 — were recently reported to have *in vivo* efficacy in animal models of HSV-1 and HSV-2 infection[16,17]. These compounds seem to function by enhancing the affinity of the helicase–primase complex for the HSV DNA. This complex comprises three viral proteins — the HSV UL5, UL8 and UL52 gene products (FIG. 3) — which together unwind the double-stranded viral DNA and generate primers for DNA synthesis by the viral DNA polymerase. The antiviral potency of BAY 57-1293 is reported to be superior to all compounds that are currently used to treat HSV infections[18]. These data validate the further pursuit of helicase–primase inhibitors for the treatment of HSV infections.

β-herpesvirus infections

Among the β-herpesviruses — HCMV, human herpesvirus type 6 (HHV-6), and human herpesvirus type 7 (HHV-7) — HCMV is associated with the primary infections CMV mononucleosis and congenital cytomegalic inclusion disease, as well as recurrent infections, such as pneumonitis, hepatitis, retinitis, encephalitis and colitis in immunocompromised hosts.

Five compounds have been licensed to treat HCMV infections — ganciclovir, its oral prodrug valganciclovir, foscarnet, cidofovir and fomivirsen. With the exception of fomivirsen, which targets HCMV immediate-early mRNA, these compounds target the viral DNA polymerase. Ganciclovir must first be phosphorylated by the HCMV-encoded protein kinase, the UL97 gene product (FIG. 2), which is also the main site for mutations that engender resistance towards this compound.

The available anti-HCMV drugs have several drawbacks that limit their clinical utility. Fomivirsen must be injected intraocularly (that is, intravitreally), foscarnet must be given intravenously three times daily and cidofovir is also administered intravenously, albeit once weekly or every other week. Nephrotoxicity is the dose-limiting factor for cidofovir and foscarnet, whereas bone-marrow suppression that results in granulocytopoenia and thrombocytopoenia is the most common toxic side effect seen with ganciclovir.

Taken together, these considerations justify the search for new anti-HCMV agents that are less toxic and/or more effective. A possible lead compound is 2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV423), which was recently shown to have potent *in vitro* activity against a wide range of HCMV reference strains and clinical isolates, including those that had acquired resistance to ganciclovir, foscarnet or cidofovir[19]. CMV423 targets a step of the viral replication cycle before the DNA polymerase step and probably coincides with immediate-early antigen expression.

The 'non-nucleoside' 4-hydroxyquinoline carboxamides (PNU-181465)[20] and 4-oxo-dihydroquinolines (PNU-182171 and PNU-183792)[21] interact with the β-herpesvirus DNA polymerase. These compounds do not inhibit the cellular DNA polymerases (α and δ) or the mitochondrial DNA polymerase γ. HSV-1 and HSV-2 viruses that are resistant to these compounds have been found to have a single amino acid change of valine to alanine within conserved domain III of the HSV-1 and HSV-2 DNA polymerases. This valine residue (V823 in HSV-1) is conserved in the DNA polymerases of six of the eight human herpesviruses and seems to have a crucial role in the observed anti-herpesvirus effects of these compounds. The DNA polymerase in HHV-6 contains an alanine at this position and accordingly is not inhibited.

The HCMV terminase, which comprises the UL89 and UL56 gene products, is a target for chemotherapeutic intervention. Together, these gene products cleave the viral high-molecular-weight DNA concatamers into unit-length genomes and package these monomeric genomes into viral procapsids. Intervention at this stage is expected to block viral DNA cleavage and packaging, leading to an accumulation of empty procapsids and unprocessed concatameric DNA, as has been demonstrated with the (naphthylsulphonylamino)phenylpropanamide BAY 38-4766 (REF. 22). BAY 38-4766 targets a viral DNA maturation step that does not occur in uninfected eukaryotic cells, so targeting the viral terminase is an attractive strategy to combat HCMV infections. In addition to BAY 38-4766, two benzimidazole ribonucleosides — 1-(β-D-ribofuranosyl)-2-bromo-5,6-dichlorobenzimidazole (BDCRB) and 1-(β-D-ribofuranosyl)-2,5,6-dichlorobenzimidazole (TCRB) — have also been shown to inhibit cleavage and encapsidation of viral DNA, and amino acid mutations in both the UL89 and UL56 gene products have been identified in resistant strains of HCMV[23].

Maribavir (or 1263W94) is another benzimidazole ribonucleoside, in which, in addition to a switch from a D to L configuration, the halogen at position 2 of the benzimidazole is replaced by an isopropylamine, and this compound also has prominent anti-HCMV activity. In

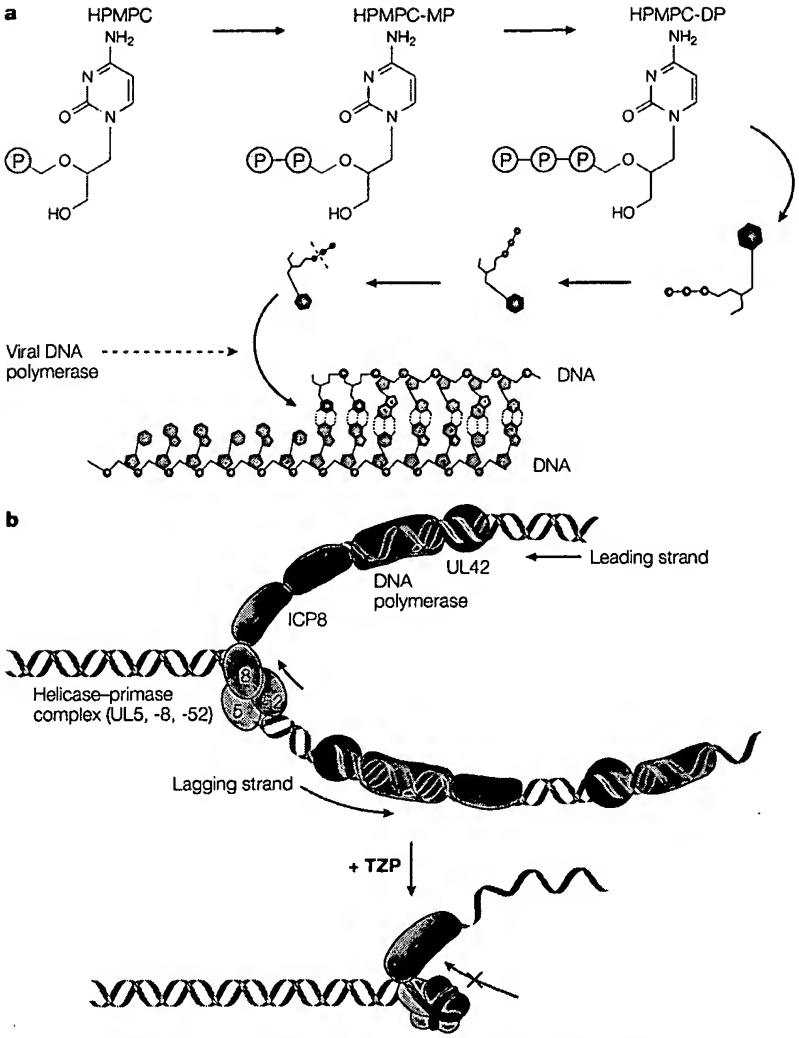

Figure 3 | **The mechanism of action of cidofovir and HSV helicase–primase complex inhibitors. a** | The mechanism of antiviral action of cidofovir (HPMPC). As an acyclic nucleoside phosphonate, cidofovir is not dependent on activation by a virus-encoded enzyme for activity. Two phosphorylations are required for activity. Reproduced with permission from REF. 1 © *Nature Rev. Drug Discov.* (2002) Nature Publishing Group. **b** | The mechanism of inhibition of DNA synthesis by HSV helicase–primase complex inhibitors. The helicase–primase complex comprises the viral gene products UL5, -8 and -52. This complex unwinds HSV DNA at the replication fork and primes DNA synthesis at both the lagging and the leading strands. The single-stranded-DNA-binding protein ICP8 binds to single-stranded template DNA. HSV DNA polymerase and its accessory protein UL42, promote leading- and lagging-strand DNA synthesis. The TZP (amino-thiazolylphenyl)-containing compounds (red band) enhance binding of the UL5 and UL52 subunits of the helicase–primase complex to both the leading- and the lagging-strand DNA, resulting in inhibition of helicase activity, primase activity and viral DNA synthesis. Modified with permission from REF. 15 © *Nature Medicine* (2002) Nature Publishing Group.

contrast to BDCRB and TCRB, however, maribavir seems to target the UL97 protein kinase[24]. The UL97 gene product has recently been shown to be responsible for the release of HCMV nucleocapsids from the nucleus[25], which means that maribavir targets a stage in the viral life cycle that follows viral DNA maturation and packaging. Preclinical pharmacokinetic and toxicological studies with maribavir have shown that it has a favourable safety profile and excellent oral bioavailability[26], and Phase I/II dose-escalation trials in HIV-infected men with asymptomatic HCMV shedding have indicated that maribavir is rapidly absorbed following oral dosing and achieved marked reductions in HCMV titre in semen[27].

Primary HHV-6 infection, which generally occurs before the patient is 2 years old, is associated with exanthema subitum, and reactivation at a later age is frequent in immunocompromised individuals, particularly bone marrow or solid-organ transplant recipients. At present there is no standardized antiviral treatment for HHV-6 infections. The most potent compounds with the highest antiviral selectivity against HHV-6 are foscarnet, S2242 [2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine] (which is the N^7-isomeric form of 6-deoxyganciclovir[28] and has excellent activity against HHV-6), A-5021 and cidofovir. For HHV-7, which has not been proven to be linked to any disease, except possibly exanthema subitum, the most potent compounds proved to be S2242, cidofovir and foscarnet[28].

γ-herpesvirus infections

Among the γ-herpesviruses, Epstein-Barr virus (EBV) is responsible for infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, lymphoproliferative syndrome, opportunistic B-cell lymphoma and oral hairy leukoplakia. Kaposi's sarcoma-associated virus (KSHV; which is also known as HHV-8) is associated with Kaposi's sarcoma, primary effusion lymphoma and Multicentric Castleman's disease.

Although several of the aforementioned licensed anti-herpetic drugs, such as acyclovir, BVDU and cidofovir, have proven to be effective against the *in vitro* replication of EBV and, in the case of cidofovir, its clinical manifestations (that is, oral hairy leukoplakia), none of these antiviral drugs has been licensed for the treatment of EBV infections. Recently, BDCRB and maribavir were shown to be active against EBV[29].

For the treatment of HHV-8 infection or associated diseases, several drug candidates have been identified, including HPMPA [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine], cidofovir, S2242 and ganciclovir[28]. Also, 4-oxo-dihydroquinolines such as PNU-183792 can inhibit HHV-8 replication due to a direct interaction with the HHV-8 DNA polymerase[21]. It has not yet been established whether any of the compounds that are effective against HHV-8 replication in cell culture *in vitro* will be efficacious in the treatment of HHV-8-associated diseases *in vivo*.

Poxvirus infections

The family of poxviridae includes orthopoxviruses (such as variola, vaccinia, cowpox, monkeypox and camelpox), parapoxviruses (such as orf) and molluscciviruses (molluscum contagiosum virus). The last natural case of smallpox, which is caused by variola virus, occurred in Somalia in 1977, and in 1980 the World Health Organization (WHO) declared smallpox to be officially eradicated. Since then, the only known stocks of variola virus have been held in Atlanta, USA, at the Centers for Disease Control (CDC), and in Koltsovo, Russia, in the State Research Centre of Virology and Biotechnology (VECTOR). If illegally

preserved stocks of variola virus were ever used for biological and/or terrorist purposes in a highly mobile and susceptible population, the results could be catastrophic. In fact, variola virus could be considered an 'ideal' bioterrorist weapon for several reasons[30]: it is highly transmissible by the aerosol route from infected to susceptible persons; the civilian populations of most countries contain a high proportion of susceptible (unvaccinated) persons; smallpox is associated with high morbidity and ~30% mortality; the initial diagnosis of a disease that has not been seen for 20 years would be difficult; and, at present, other than the vaccinia-based vaccine, which might be effective in the first few days post-infection, there is no formally approved drug for the treatment of smallpox.

The first antiviral compound to be used in the treatment and prophylaxis of smallpox, treatment of complications after smallpox vaccination and the treatment of eczema vaccinatum and vaccinia gangrenosa was methisazone (*N*-methylisatin 3-thiosemicarbazone)[31]. Severe side effects were reported[31] and follow-up studies[32] revealed that methisazone has *in vitro* activity against monkeypox virus and variola virus only at concentrations that are too high (~50 $\mu g\ ml^{-1}$) to be therapeutically meaningful.

Yet, several potential antiviral therapeutics have proven to be active against orthopoxvirus infections, both in cell culture[33] and in animal models[34], at concentrations and/or doses that could be used therapeutically. Several potential therapeutic strategies have been designed to target cellular enzymes. These enzymes include inosine 5′-monophosphate (IMP) dehydrogenase, which is responsible for the conversion of IMP to XMP (xanthosine 5′-monophosphate), an important step in the *de novo* biosynthesis of GTP; S-adenosylhomocysteine (SAH) hydrolase, the enzyme responsible for the hydrolysis of SAH, which is the product-inhibitor of S-adenosylmethionine (SAM)-dependent methylation reactions such as those involved in the maturation of viral mRNAs; oritidine 5′-phosphate (OMP) decarboxylase, the enzyme that is responsible for the conversion of OMP to UMP (uridine 5′-monophosphate), which is an important reaction in the *de novo* biosynthesis of UTP (uridine 5′-triphosphate); cytosine 5′-triphosphate (CTP) synthetase, which converts UTP to CTP; and viral enzymes such as the poxviral DNA polymerase[35,36].

Several nucleoside and nucleotide analogues have been identified as potent anti-poxvirus agents. The nucleoside analogues include S2242 and 8-methyladenosine, whereas the nucleotide analogues include cidofovir, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine (HPMPDAP) and (*S*)-6-(3-hydroxy-2-phosphonylmethoxypropyl)oxy-2,4-diaminopyrimidine (HPMPO-DAPy). These compounds have been shown to be effective in various animal models of poxvirus infections[37]. Cidofovir can protect mice from a lethal respiratory infection with either vaccinia or cowpox, even when administered as a single systemic (intraperitoneal) or intranasal (aerosolized) dose[38], and is effective in the treatment of vaccinia virus infection in severe combined immune deficiency (SCID) mice.

In humans, anecdotal reports suggest that both topically and intravenously administered cidofovir can successfully treat recalcitrant molluscum contagiosum and orf infections in immunocompromised patients[38]. Cidofovir is licensed for clinical use (in the treatment of CMV retinitis in AIDS patients) by intravenous injection. However, it could also be formulated for topical administration (for example, as a gel or cream) or for oral administration in prodrug form. The 1-*O*-hexadecyloxypropyl derivative (HDP-cidofovir), which has increased anti-poxvirus activity relative to cidofovir[39] owing to facilitated cellular uptake[40], is under investigation as an oral prodrug.

Although the ultimate proof — activity against smallpox in humans — has not been (and, for obvious reasons, cannot be) provided, from the above discussion it can be inferred that cidofovir could be effective in the therapy and short-term prophylaxis of smallpox and related poxvirus infections (that is, monkeypox) in humans, as well as in the treatment of complications of vaccinia that can arise in immunocompromised patients who are inadvertently inoculated with the smallpox vaccine (vaccinia). A murine model that mimics progressive and disseminated vaccinia in humans has recently been developed[41]. In this model (athymic nude mice inoculated intracutaneously with vaccinia virus), systemic treatment with cidofovir when disseminated vaccinia developed caused the lesions to heal and regress. In most of the animals treated in this way the lesions completely (or almost completely) disappeared within 10–15 days of the start of therapy[41]. These observations have implications for the therapy of complications of smallpox vaccination.

Hepadnavirus infections

More than 350 million people worldwide are chronically infected with the hepadnavirus HBV, and complications of chronic hepatitis B infection, such as cirrhosis, hepatocellular carcinoma and end-stage liver disease, account for approximately 1 million deaths each year. The drugs that have been formally approved for the treatment of chronic hepatitis B are interferon (IFN)-α, lamivudine (3TC) and adefovir dipivoxil [bis(POM)PMEA]. However, IFN treatment is compounded by side effects, such as influenza-like symptoms, anorexia and depression, which require dose adjustment or even discontinuation of therapy. In contrast with IFN, which must be given by parenteral administration, lamivudine can be administered orally and is well tolerated, but leads to resistance in up to 39% of patients after one year of therapy and 66% after four years of therapy.

Adefovir dipivoxil is the oral prodrug of adefovir (PMEA), which, after intracellular conversion to the diphosphate form, acts as a competitive inhibitor or alternative substrate for HBV reverse transcriptase and, when incorporated into the DNA, acts as a chain terminator, thereby preventing DNA elongation[42] (FIG. 4). In patients with chronic HBV infection who were either positive[43] or negative[44] for hepatitis B e antigen, 48 weeks of treatment with a dose of adefovir dipivoxil as low as 10 mg day^{-1} resulted in significant improvement in

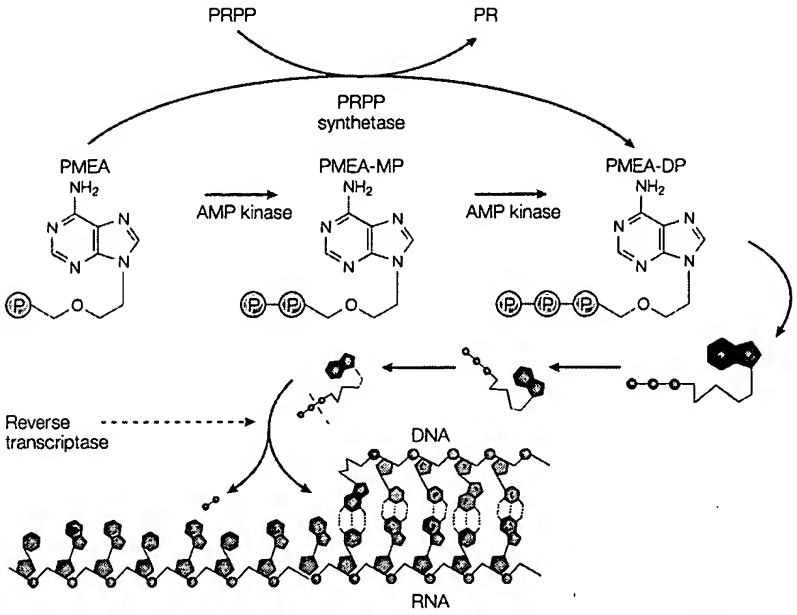

Figure 4 | **The mechanism of action of adefovir (PMEA).** After intracellular conversion to the diphosphate form, adefovir acts as a competitive inhibitor for HBV reverse transcriptase. When adefovir is incorporated into the DNA it acts as a chain terminator, thereby preventing DNA elongation. Reproduced with permission from REF.1 © *Nature Rev. Drug Discov.* (2002) Nature Publishing Group.

histological liver abnormalities, as well as a significant reduction in serum HBV DNA and alanine aminotransferase concentrations. In these trials, the safety profile of adefovir dipivoxil was similar to that of the placebo, and no HBV polymerase mutations associated with resistance to adefovir were identified. In fact, adefovir dipivoxil proved effective in HBV-infected patients that had developed resistance to lamivudine[42]. Resistance to adefovir might eventually develop but at a much slower rate and lower frequency than has been observed for lamivudine.

Entecavir and the β-L-nucleosides L-FMAU (1-(2-fluoro-5-methyl-β-L-arabinosyl)uracil; clevudine), β-L-thymidine (L-dT), the valine ester of β-L-2′-deoxycytidine (val-L-dC) and emtricitabine ((–)FTC) are under clinical development[45] for the treatment of chronic hepatitis B. Emtricitabine is the 5-fluoro-substituted counterpart of lamivudine and has already been licensed for the treatment of HIV infections. Owing to its structural similarity to lamivudine, it is thought that emtricitabine might engender the same resistance mutation (substitution of a methionine residue with leucine or valine residues) as is seen for lamivudine, and thereby produce cross-resistance to lamivudine.

Entecavir is a 2′-deoxyguanosine analogue and has *in vitro* and *in vivo* potency that seem to be greater than that of lamivudine — in patients with chronic hepatitis B infection it has proven efficacious at doses as low as 0.1 and 0.5 mg day^{-1} (REF. 46). Potent efficacy has also been demonstrated with entecavir against woodchuck hepatitis virus (WHV) in woodchucks and duck hepatitis B virus (DHBV) in ducks[47]. For entecavir to be active against hepadnavirus replication, it must — as for all nucleoside analogues — be converted intracellularly to its 5′-triphosphate form, which, in the case of entecavir, would then compete with dGTP for the viral DNA polymerase. Intracellular entecavir 5′-triphosphate can be expected to accumulate at concentrations that are inhibitory to both wild-type and 3TC-resistant HBV DNA polymerase[48].

Like entecavir, L-FMAU has proven to be a potent inhibitor of both DHBV and WHV replication in acutely infected ducks and chronically infected woodchucks, respectively[49]. At the highest dose administered (10 mg kg^{-1} day^{-1}), treatment with L-FMAU led to a reduction in the levels of covalently closed circular WHV DNA and, concomitantly with this, long-lasting suppression of viraemia after withdrawal of therapy[49]. Also, L-FMAU treatment followed by therapeutic vaccination to break immune tolerance has been advocated as a strategy to control chronic HBV infection in humans[50].

The β-L-nucleoside analogues L-FMAU, L-dT and L-dC must also be phosphorylated to their 5′-triphosphate form to interact with HBV DNA polymerase. In fact, L-dT and L-dC have been shown to be rapidly and extensively phosphorylated in both hepatoma cells and primary human hepatocytes[51]. The 3-phosphoglycerate kinase — a glycolytic enzyme that uses 1,3-bisphosphoglycerate as a phosphate donor to generate ATP during glycolysis — would have an important role in the phosphorylation of the L-nucleosides (diphosphates) to their 5′-triphosphate derivatives[52].

It is noteworthy that adefovir dipivoxil — a drug that has now been approved worldwide for the treatment of chronic HBV infection — might be a promising adjunctive therapy against anthrax and other human diseases caused by pathogenic bacteria that secrete adenylyl cyclase toxins, such as *Bordetella pertussis*, *Pseudomonas aeruginosa* and *Yersinia pestis*[53]. Indeed, the active cellular metabolite of adefovir, adefovir diphosphate (PMEADP), was recently shown to inhibit the adenylyl cyclase activity of the oedema factor (K_i of 27 nM), which is an important virulence factor in the pathogenesis of anthrax, especially during the early stages of the *Bacillus anthracis* infection where it contributes to both toxaemia and bacteraemia.

Picornavirus infections

The picornaviruses include the enteroviruses (such as poliovirus, coxsackieviruses A and B, echovirus and hepatitis A virus) and rhinoviruses. Rhinoviruses are the main cause of the common cold, whereas enterovirus infections result in a myriad of disease syndromes, including viral meningitis and viral respiratory infections. Of these viruses, poliovirus has historically received most attention. After a successful worldwide vaccination campaign, the only countries in which polio still remains endemic are Niger, Nigeria, Egypt, India, Pakistan and Afghanistan. Hepatitis A can also be controlled sufficiently by the use of a killed virus vaccine. No vaccines are available for prevention of coxsackieviruses A and B, echovirus and rhinovirus infections.

The compound that has been most extensively studied against both entero- and rhinoviruses is pleconaril.

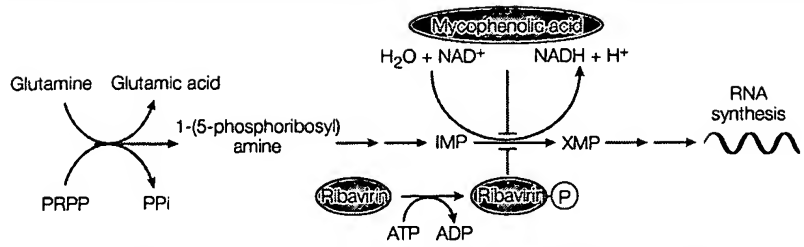

Figure 5 | **The mechanism of action of ribavirin and mycophenolic acid, the active component of mycophenolate mofetil.** Both these compounds block RNA synthesis by inhibiting the action of inosine 5′-monophosphate (IMP) dehydrogenase — this blocks the conversion of IMP to XMP (xanthosine 5′-monophosphate) and thereby stops GTP and, consequently, RNA synthesis.

This compound binds to a hydrophobic pocket beneath the 'canyon floor' of the VP1 capsid protein of picornaviruses[54], thereby 'freezing' the viral capsid and preventing its uncoating from the viral RNA genome. Pleconaril is orally bioavailable and achieves plasma concentrations greater than those that are required to inhibit 90% of clinical rhino- and enteroviral isolates *in vitro*. In addition, in the central nervous system (CNS) and nasal secretions the drug is present at concentrations several fold higher than in plasma — a highly desirable feature for an antiviral drug targeted towards viruses that are known to cause CNS and upper-respiratory-tract infections[55]. The clinical efficacy of pleconaril has been assessed in experimentally induced enterovirus (coxsackievirus A21) respiratory infections in adult volunteers[56] and, on a compassionate basis[57], against potentially life-threatening enterovirus infections. When administered three-times daily, pleconaril reduced the duration and severity of picornavirus-associated viral respiratory illness in adolescents and adults[58,59].

Coxsackie B viruses are thought to be the main aetiological agents of viral myocarditis, which is a common cause of idiopathic dilated cardiomyopathy — a severe pathological condition that often requires heart transplantation — and are therefore targets for chemotherapy. The immunosuppressive agent mycophenolate mofetil inhibits the development of coxsackie B3 virus-induced myocarditis in C3H mice[60]. Mycophenolic acid (MPA), the active component of mycophenolate mofetil, is a potent inhibitor of IMP dehydrogenase and its antiviral and immunosuppressive effects can be attributed to this interaction, which reduces the supply of GTP (FIG. 5), and therefore, RNA synthesis. The beneficial effect of MPA cannot be ascribed to inhibition of viral replication, as the titre of infectious virus and viral RNA in heart tissue was increased in the mycophenolate-mofetil-treated animals compared with untreated animals[60].

A more marked inhibitory effect on the development of coxsackie B3-virus-induced myocarditis, corroborated by a marked reduction in the virus titres in the heart, was obtained with the IFN inducers poly(I)·poly(C) and poly(I)·poly(C_{12}U) (also known as ampligen) and, to a lesser extent, with IFN-α2b and pegylated IFN-α2b[61]. Even when the start of treatment with poly(I)·poly(C_{12}U) was delayed until two days post-infection, when lesions had already appeared in the untreated control animals, a marked protective effect on the development of viral myocarditis (assessed six days post-infection) was observed. A combination of an inhibitor of viral replication (such as ampligen) and an immunosuppressant (such as mycophenolate mofetil) could be an ideal treatment strategy for viral myocarditis. How to implement such a treatment regimen in the clinical setting remains to be addressed.

Flavivirus infections

The genus *Flavivirus* contains more than 70 species, many of which cause disease in humans. Severe flavivirus infections are generally characterized by encephalitis or haemorrhagic symptoms. Mortality rates vary from 1–2% (in the cases of Central European encephalitis virus) to 30–40% (in the case of Japanese encephalitis virus and tick-borne encephalitis virus, which was previously known as Russian Spring and Summer encephalitis virus). Other important flaviviruses include yellow fever virus, dengue virus, West Nile virus, St Louis encephalitis virus and Murray Valley encephalitis virus. Although feared as a possible bioterrorist weapon, the development of tick-borne flaviviruses as bioweapons might not be practical, as large numbers of infected ticks would be required and it would be difficult to arrange for them to be infected and ready to feed when delivered as weapons[62].

The prospects for the therapy of flavivirus infections are not encouraging[63]. There are some compounds — 6-azauridine, cyclopentenylcytosine, MPA and pyrazofurin — that have activity against West Nile virus[64]. Ribavirin has only weak activity against flaviviruses. The use of IFN and IFN inducers might be possible, but, in general, this treatment should be started before or very shortly after infection to have any beneficial effect. An experimental flavivirus encephalitis model has been developed based on infection of hamsters with the murine Modoc virus[65]; during the acute phase, the infection is associated with flaccid paralysis and the neurological sequelae that can develop are similar to those that have been observed in survivors of Japanese encephalitis[65]. This model should be suitable for the evaluation of anti-flavivirus therapies. At present, IFN-α2b, whether pegylated or not, and IFN inducers (poly(I)·poly(C) and ampligen) offer the greatest potential for activity in this model, as they have been shown to significantly delay virus-induced morbidity (paralysis) and mortality (due to progressive encephalitis) in a related model with Modoc virus in SCID mice[66]. It is noteworthy that ribavirin did not provide any beneficial effect in this model, whether given alone or in combination with IFN.

Arenavirus infections

Of the 23 arenavirus species that are known, five are associated with viral haemorrhagic fevers — Lassa, Junin, Machupo, Guanarito and Sabia[67]. These viruses are included in the CDC Category A Pathogen List. It is gratifying to note that, as demonstrated with Tacaribe virus and an attenuated Junin virus strain, *in vitro*

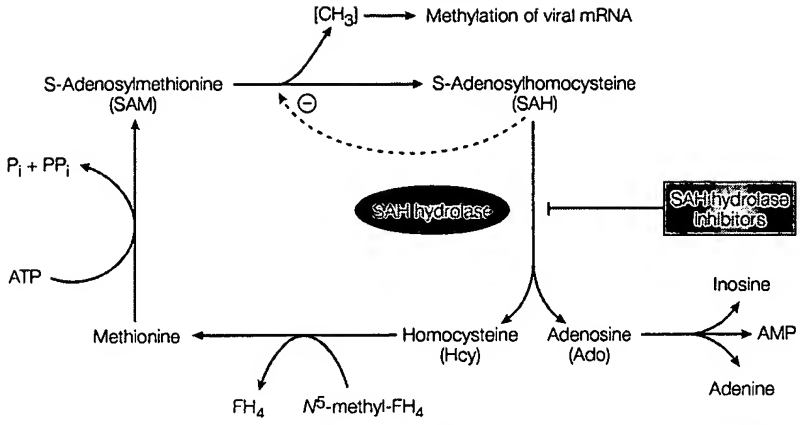

Figure 6 | **The mechanism of action of adenosine analogues such as 3-deazaneplanocin A.** These compounds inhibit the action of S-adenosylhomocysteine (SAH) hydrolase, which hydrolyses SAH, the product-inhibitor of S-adenosylmethionine (SAM)-dependent methylation reactions. Inhibiting these reactions can affect transcription.

arenavirus replication is susceptible to several compounds, including adenosine analogues (for example, SAH hydrolase inhibitors such as 3-deazaneplanocin A), cytidine analogues (for example, cyclopentenyl cytosine), guanosine analogues (for example, IMP-dehydrogenase inhibitors such as ribavirin) and sulphated polysaccharides (for example, dextran sulphate)[68].

Ribavirin has proven to be effective in the post-exposure prophylaxis and therapy of experimental arenavirus infections in animal models, and anecdotal reports suggest that it might also be effective in the treatment of arenavirus infections (Machupo and Sabia viruses) in humans[67]. The most convincing evidence for the efficacy of ribavirin was obtained in the case of Lassa fever, where it was shown to reduce the case-fatality rate, irrespective of the time point in the illness at which treatment was started[69].

Bunya- and togavirus infections

Several togaviruses and bunyaviruses have been described as potential bioterrorism agents — for example, the togaviruses Venezuelan equine encephalitis virus, Eastern equine encephalitis virus and Western equine encephalitis virus, and the bunyaviruses Rift Valley fever virus, Crimean–Congo haemorrhagic fever virus and hantaviruses such as Hantaan virus[70]. However, hantaviruses are unlikely candidates for biological warfare purposes as they are difficult to isolate (and grow) in cell culture, they are not transmitted between humans and there is no evidence that they are infectious by aerosol[71]. Crimean–Congo haemorrhagic fever virus, however, can be readily cultivated, is highly infectious (although so far there is no evidence that it is infectious in aerosol form) and is easily transmitted between humans, giving rise to local epidemics and even nosocomial infections. The case-fatality rate associated with Crimean–Congo haemorrhagic virus is ~30%, which is higher than that of most other viral haemorrhagic fevers[71].

Bunyaviruses are generally sensitive to ribavirin, and this has also been demonstrated in experimental animal models[70]. IFN and IFN inducers have also proved effective in the treatment of experimental bunyavirus infection, if, as is usual for these compounds, they are administered as early as possible after infection. As for flavivirus infections, ribavirin is of no use in the treatment of togavirus infections. For these infections, IFN (whether pegylated or not) and IFN inducers (such as ampligen) are the recommended therapeutic agents[70].

Rhabdo- and filovirus infections

Rhabdoviruses (such as Rabies virus) and filoviruses (such as Ebola and Marburg viruses) are among the most deadly viruses to infect humans. Rabies is almost invariably fatal, as illustrated by a recent case report[72]; however, rabies can be contained by repeated administration of specific immunoglobulin and the use of a killed rabies vaccine as soon as possible after the infection has taken place. No vaccine is available for either Ebola or Marburg infections and these viruses are classified as Category A Pathogens. Filoviruses are highly infectious by the airborne route, but can also be transmitted between humans through direct contact with virus-containing body fluids. Although filoviruses could be more difficult for potential bioterrorists to acquire than other biological agents such as *B. anthracis*, their reputation for causing deadly disease might make the effort required seem worthwhile[73].

Specific immunoglobulin or IFN-α2b are of only limited value in the treatment of experimental Ebola virus infections — for example, rhesus macaques that were treated from the day of infection with Ebola (Zaire) virus experienced a delay of only one day in the onset of illness, viraemia and death[74]. No antiviral drugs that are currently in clinical use, including ribavirin, provide any protection against filoviruses[73]. The most promising therapeutic strategy might be based on the use of SAH hydrolase inhibitors such as 3-deazaneplanocin A. As already described, SAH hydrolase inhibitors interfere with SAM-dependent methylation reactions (FIG. 6) such as those involved in the 'capping' of viral mRNA.

Some viruses, including rhabdoviruses such as vesicular stomatitis virus (VSV), rely on mRNA 'capping', as they are particularly sensitive to inhibition by SAH hydrolase inhibitors[75]. Biochemically, filoviruses are similar to rhabdoviruses — both require 5′-capping of the mRNAs — and, therefore, it could be logically deduced that SAH hydrolase inhibitors such as neplanocin A and 3-deazaneplanocin A, which are highly active both *in vitro* and *in vivo* against VSV[76], would also be effective in the treatment of Ebola virus infections.

In fact, when administered as a single dose of 1 mg kg^{-1} on the first or second day after an Ebola Zaire virus infection in mice, 3-deazaneplanocin A reduced peak viraemia by more than 1,000-fold compared with mock-treated controls, and most or all the animals survived[77]. This protective effect was accompanied, and probably mediated, by the production of high

concentrations of IFN-α in the Ebola virus-infected mice[78]. It can be hypothesized that, by blocking the 5'-capping of the nascent (+)RNA viral strands, 3-deazaneplanocin A prevented the dissociation of these strands from the viral (–)RNA template, thereby leading to an accumulation of replicative intermediates. These replicative intermediates — composed of dsRNA stretches — could then induce the production of high concentrations of IFN[79].

Hepacivirus infections

It is estimated that more than 170 million people worldwide are infected with the hepacivirus hepatitis C virus (HCV), which is a bloodborne virus that is often sub-clinical but which, in up to 85% of cases, leads to a chronic infection that ultimately results in liver fibrosis (cirrhosis), hepatic failure or hepatocellular carcinoma. HCV infection is the most common cause of hepatocellular carcinoma and the main reason for liver transplantation among adults in western countries. The development of effective anti-HCV therapeutics continues to be a daunting challenge owing to the absence of adequate animal models and cell-culture systems for evaluating propagation of the virus and its inhibitors[80].

At present, the recommended (and approved) therapy for chronic HCV infections consists of pegylated IFN-α2a combined with ribavirin. This therapy is associated with a sustained viral response rate of ~50% among patients infected with HCV genotype 1 and of ~80% in patients infected with HCV of another genotype. Treatment with pegylated IFN-α2a and ribavirin can be individualized by genotype[81]. Patients that are infected with HCV genotype 1 require treatment for 48 weeks[81] (or longer[82]), whereas patients that are infected with HCV genotypes 2 or 3 can be treated for 24 weeks. In addition, for the latter group, the dose of ribavirin can be reduced (from 1,000 or 1,200 mg day^{-1} to 800 or even 600 mg day^{-1}). Lowering the duration of therapy with a combination of pegylated IFN-α and ribavirin is not a trivial issue owing to both the cost of therapy and its associated toxicities (flu-like syndrome, depression and alterations in red blood cell counts).

Although IFN is generally an immunomodulatory agent and ribavirin is an antiviral agent, when the two agents are used in combination against hepatitis C they appear to act the other way around. Recent work has focused on the development of compounds that interfere with the non-structural (NS) protein-associated NTPase/helicase, serine protease and RNA-dependent RNA polymerase (RNA replicase) activities of HCV. Halogenated benzimidazoles and benzotriazoles have been proposed to be inhibitors of the HCV NTPase/helicase[83] but whether they also inhibit HCV replication remains to be determined. Recently, a NS3 protease inhibitor (BILN 2061) was reported to reduce the plasma concentrations of HCV RNA when administered orally for 2 days to patients who were infected with HCV genotype 1, thereby providing proof-of-efficacy for the use of HCV NS3 protease inhibitors in humans[84].

An attractive approach for the development of HCV inhibitors is to target the NS5B RNA-dependent RNA polymerase (RdRp). The impetus for such an approach comes from the fact that VP 32947 or 3-[((2-dipropylamino)ethyl)thio]-5H-1,2,4-triazino(5,6-b)indole was found to suppress the replication of bovine viral diarrhoea virus (BVDV) through an inhibitory effect on the NS5B RdRp[85]. Infections with this pestivirus have an economic impact, but can also be considered as a surrogate virus for HCV. Other compounds that have been identified as highly selective inhibitors of BVDV replication owing to a specific inhibitory effect on the BVDV RNA replicase are compound 1453 (REF. 86) and compound '22' (REF. 87). Although these compounds, in their own right, could be pursued for the treatment of pestivirus infections in domesticated livestock, they could also be model compounds for the development of non-nucleoside HCV RdRp inhibitors. As non-nucleoside RdRp inhibitors such as compound '22' are active at nanomolar concentrations[87], they seem, at first glance, much more potent than the (ribo)nucleoside analogues, such as N^4-hydroxycytidine[88], that have been reported to block the replication of BVDV and HCV.

Orthomyxovirus infections

Of the orthomyxoviruses, influenza A and influenza B viruses cause epidemics in humans. Influenza A viruses, which have been isolated from a wide variety of avian and mammalian species, can cause widespread human epidemics or pandemics with high mortality rates because these viruses are readily and rapidly transmitted between humans by the aerosol route. Whereas influenza B virus only undergoes antigenic drift based on relatively minor changes (transition and/or transversion mutations) in the viral surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), influenza A virus is prone to both antigenic drift and antigenic shift, the latter resulting from major antigenic changes owing to reassortment of genomic fragments between influenza viruses of different animal species.

The high virulence of some influenza A virus strains, such as H5N1, which emerged in Hong Kong in 1997, and the fact that lethal influenza A viruses can be generated in the laboratory by reverse genetics, have accentuated the fear of influenza A viruses being used as bioterrorist weapons[89]. Additionally, highly pathogenic avian influenza A viruses — for example, subtype H7N7 — that are responsible for fowl plague in poultry, can be transmitted to people who handle infected poultry and be further transmitted from person to person[90]; a fatal course of pneumonia in association with acute respiratory distress syndrome has been noted in an individual infected with the avian H7N7 virus[91].

For many years, amantadine and rimantadine have been used for the prophylaxis and therapy of influenza A virus infections, but they have not gained wide acceptance for three reasons. First, these agents do not have activity against influenza B viruses, as these viruses lack the matrix protein M2 that determines the anti-influenza-virus activity of amantadine and rimantadine; second, the prospect of rapid emergence of drug-resistant virus

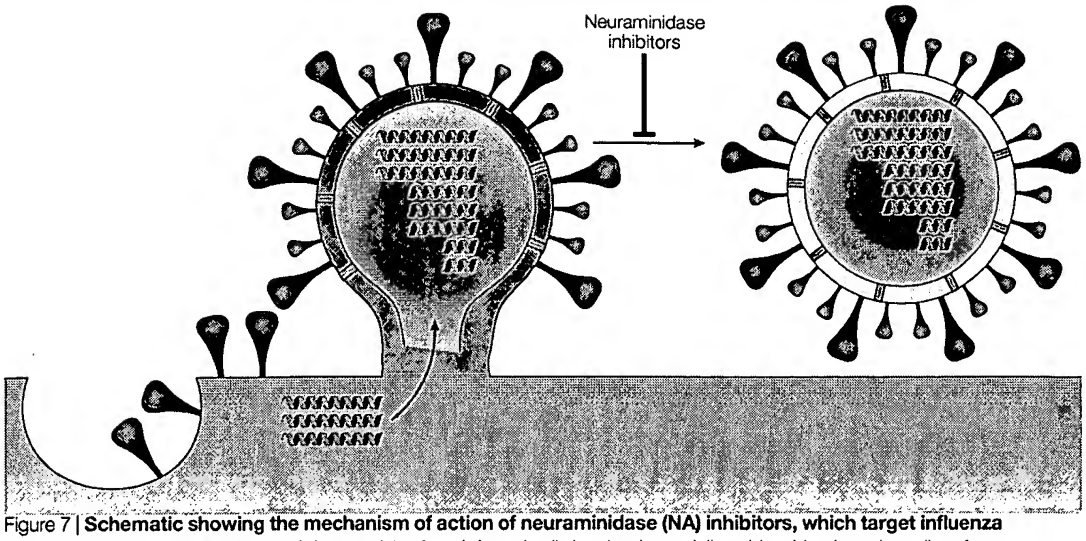

Figure 7 | **Schematic showing the mechanism of action of neuraminidase (NA) inhibitors, which target influenza viruses.** NA facilitates the release of virus particles from infected cells by cleaving a sialic acid residue from the cell-surface glycoprotein. By blocking this reaction, NA inhibitors prevent the release of virus.

mutants; and third, possible side effects affecting the CNS, which have been particularly documented for amantadine.

Given their specificity for influenza virus strains that are already circulating, influenza vaccines are likely to be of limited value against a newly emerging influenza strain, whether occurring naturally or as a bioterrorist weapon. In this case, antiviral drugs that are directed at functions shared by as many influenza strains as possible would constitute the best line of defence[89]. The NA inhibitors zanamivir[92] and oseltamivir[93] meet these requirements. These compounds prevent the removal of the sialic acid (*N*-acetylneuraminic acid) residue from the glycopeptide receptor (FIG. 7) by the viral NA, which would otherwise allow the virus particles to be released from the infected cell (and spread to neighbouring cells). Both have been licensed for the treatment and prophylaxis of influenza virus infections, and it would be advisable to have stockpiles of these compounds (particularly oseltamivir because it can be conveniently administered as capsules) to be used in case of an influenza virus outbreak or attack.

Paramyxovirus infections

The paramyxoviruses include parainfluenza 1, 2, 3, 4a and 4b, Sendai virus, mumps virus, measles virus, Hendra and Nipah viruses, and the pneumoviruses respiratory syncytial virus (RSV) and human metapneumovirus (hMPV). Parainfluenza has been little studied from either a preventative or curative viewpoint. Mumps and measles, like rubella, are now sufficiently contained by vaccination, which makes Nipah virus (and the related Hendra virus), RSV and hMPV the paramyxoviruses for which antiviral approaches are required.

Nipah virus was isolated during an outbreak of viral encephalitis in Malaysia 5 years ago and has many characteristics that would make it a potential bioterrorist weapon[94]. There is no specific antiviral treatment for Nipah virus infections.

hMPV was first isolated in 2001 from young children with respiratory-tract disease[95]. The clinical symptoms are similar to those caused by RSV, and range from upper-respiratory-tract disease to severe bronchiolitis and pneumonia. hMPV is similar to RSV in that infection usually occurs during the winter months and is common in young children, elderly people and immunocompromised individuals. In a study carried out on hospitalized patients with respiratory-tract illness, hMPV was the second-most-detected viral pathogen (RSV being the first) during two successive winter seasons[96]. There is no specific antiviral treatment for hMPV infection.

A significant number of patients who are diagnosed with influenza-like illness harbour RSV and, as influenza and RSV infections occur at approximately the same time, there is a need to distinguish between the two to prescribe specific antiviral treatment[97]. As mentioned above, specific treatment for influenza consists of the NA inhibitors (zanamivir and oseltamivir), whereas for RSV infections the only approved therapy is aerosol administration of ribavirin. In practice, however, ribavirin is rarely used owing to the technical burden of delivery by aerosol inhalation. Attempts have been made to develop RSV inhibitors that target the viral fusion (F) protein and therefore block virus–cell fusion and syncytium formation. An example is 4,4′-bis-[4,6-bis-(3-(bis-carbamoylmethyl-sulphamoyl)-phenylamino)-(1,3,5)triazin-2-ylamino]-biphenyl-2′,2′-disulphonic acid (RFI-641), which has proved to be efficacious when administered prophylactically (or up to 24 hours post-infection) by the intranasal route in mice, cotton rats and African green monkeys intranasally infected with RSV[98].

Coronavirus infections

In the past, human coronavirus infections, such as infection by 229E virus, were not considered sufficiently serious to be controlled by either vaccination or

specific antiviral therapy. This has now changed markedly with the emergence of severe acute respiratory syndrome (SARS), which has been unequivocally associated with a newly discovered coronavirus — SARS-associated coronavirus (SARS-CoV)[99–104]. The disease is mainly characterized by influenza-like symptoms, high fever, myalgia, dyspnea, lymphopoenia and lung infiltrates (pneumonia) leading to acute breathing problems, with an overall mortality rate of about 10% (in the elderly this can be as high as 50%).

The genome structure, life cycle and phylogenetic relationships of SARS-CoV have been addressed previously[105]. There are several proteins encoded by the SARS-CoV genome that could be considered targets for chemotherapeutic intervention: the spike (S) protein, the coronavirus main proteinase (3CLpro), the NTPase/helicase, the RNA-dependent RNA polymerase and, possibly, other viral-protein-mediated processes.

The coronavirus S protein mediates infection of permissive cells through interaction of its S1 domain with angiotensin-converting enzyme 2 (ACE2), which is a functional receptor for SARS-CoV[106]. A 193-amino-acid fragment of the S protein (corresponding to residues 318–510) binds ACE2 more efficiently than the full S1 domain and, in fact, the 193-residue fragment blocks S-protein-mediated infection with an IC_{50} of <10 nM (the IC_{50} of the full S1 domain is ~50 nM)[107]. Also, human monoclonal antibodies to the S1 protein block the association of SARS-CoV with ACE2, indicating that the ACE2-binding site of S1 could be a target for drug development[108]. The first small-molecular-weight inhibitor that was found to interact with the ACE2 active catalytic site, (*S,S*)-2-[1-carboxy-2-(3-(3,5-dichloro-benzyl)-3H-imidazol-4-yl)-ethylamino]-4-methyl-pentanoic acid (MLN-4760), has already been described[109]. Whether MLN-4760 inhibits SARS-CoV infection remains to be ascertained.

The coronavirus main proteinase, Mpro, also known as 3CLpro, is a target for the design of anti-SARS-CoV drugs[110]. It was proposed that compounds such as AG7088, which have proven to be active against the rhinovirus 3C proteinase, could be modified to make them active against coronaviruses[110]. A first modification of AG7088 that removed the methylene group of the *p*-fluorophenylalanine residue created KZ7088. KZ7088 has been modelled into the structure of the SARS-CoV 3Clpro protein[111], and further work in this area could advance structure-based drug design against SARS[112]. Another potential target for the development of anti-SARS agents is the SARS-CoV-associated NTPase/helicase[113].

The SARS-CoV RNA-dependent RNA polymerase is also a potential target for anti-SARS therapy[114]. This enzyme does not contain a hydrophobic pocket for non-nucleoside inhibitors such as those that have proven active against HCV polymerase or HIV-1 reverse transcriptase[114]. Of the many nucleoside analogues that are expected to target the SARS-CoV RNA polymerase and for which efficacy has been determined, only N^4-hydroxycytidine — incidentally, the same compound that has been accredited with anti-HCV activity[73] — showed activity, albeit at a low level (EC_{50} of 10 μM; selectivity index of ≥10), against SARS-CoV replication in cell culture[115].

In addition to N^4-hydroxycytidine, some calpain inhibitors (*N*-(4-fluorophenylsulphonyl)-L-valyl-L-leucinal) inhibit SARS-CoV replication (EC_{50} of 1 μM; selectivity index of ≥100)[115]. The target of the calpain inhibitors remains to be elucidated. Inhibitory effects on SARS-CoV (again with selectivity indexes of up to ~100 and EC_{50} values as low as 1 μg ml^{-1}), have been observed for a variety of compounds, including vancomycin, eremomycin and teicoplanin aglycon derivatives[116], and mannose-specific plant lectins, derived from *Galanthus nivalis* (snowdrop), *Hippeastrum* hybrid (amaryllis)[117] or *Allium porrum* (leek)[118], which might all owe their antiviral activity to an interaction with components of the viral entry machinery. Glycyrrhizin has also been shown to inhibit the replication of SARS-CoV[119], but only at concentrations (EC_{50} of 300–600 μg ml^{-1}) that could not be achieved in the target tissue or organs.

An effective agent, at least for the prophylaxis and early post-exposure management of SARS, would seem to be human IFN, either α, β or γ[120]. Pegylated IFN-α was recently shown to reduce viral replication and excretion, viral antigen expression by type 1 pneumocytes and the attendant pulmonary damage in cynomolgus macaques that were infected experimentally with SARS-CoV[121]. These preliminary results warrant further studies with pegylated IFN-α, which is commercially available, in the prophylactic or early post-exposure treatment of SARS should it re-emerge.

Reovirus infections

Rotavirus, which is associated with worldwide epidemics of viral gastrointestinal infections, is the most clinically important of the reoviruses. Although several attempts have been, and are still being, made to develop an effective vaccine for rotavirus infections, the current treatment for this infectious diarrhoea is mainly based on the administration of fluids (physiological saline) to prevent dehydration. Yet, it should be pointed out that SAH hydrolase inhibitors such as 3-deazaneplanocin A (see above) offer great promise for the treatment of reo- (or rota-) virus infections. In cell culture[76], 3-deazaneplanocin A was found to inhibit rotavirus replication at an EC_{50} of 0.04 μg ml^{-1} and a selectivity index of 10,000.

Retrovirus infections

Since the identification of HIV as the causative agent of AIDS more than 20 years ago, so many efforts have been made to keep this disease under control that 19 compounds have been formally approved as anti-HIV drugs, and they can be used in a variety of combinations[122]. They can be divided into five categories: the nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine; the nucleotide reverse transcriptase inhibitors (NtRTIs), such as tenofovir disoproxil fumarate; the non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine, delavirdine and efavirenz; the protease inhibitors (PIs),

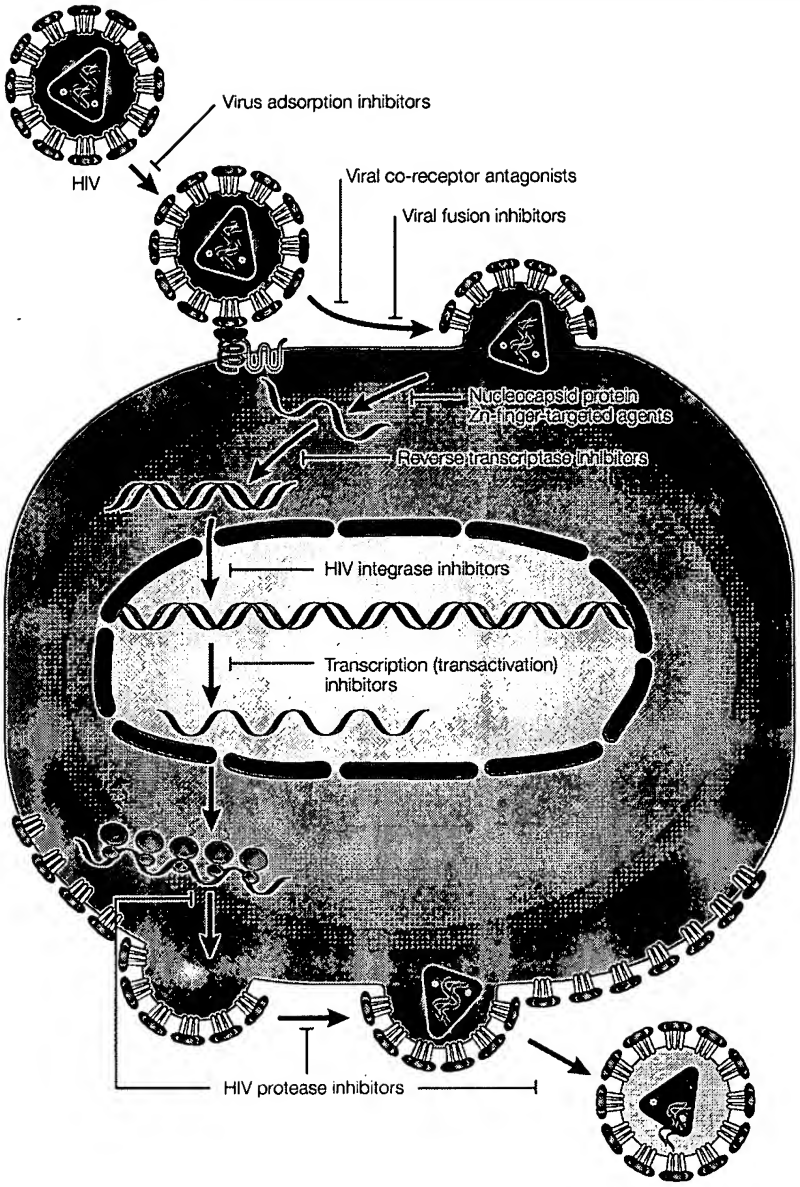

Figure 8 | **Schematic representation of the HIV life cycle, depicting the targets for anti-HIV agents.** So far, 19 compounds have been licensed for use in HIV therapy. The five main drug categories are nucleoside reverse transcriptase inhibitors, nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and fusion inhibitors. New agents include viral adsorption inhibitors and co-receptor antagonists. Modified with permission from REF.1 © *Nature Rev. Drug Discov.* (2002) Nature Publishing Group.

such as saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir and atazanavir; and enfuvirtide, which is a fusion inhibitor (FI).

In addition, various new anti-HIV agents have been described that are presently under clinical or preclinical development[123]. These new agents target either the same viral proteins as the 'old' ones — reverse transcriptase, protease or the gp41 envelope glycoprotein — which mediate fusion, or 'new', yet to be therapeutically validated, viral processes such as viral adsorption (mediated by the gp120 glycoprotein), co-receptor (CXCR4 or CCR5) usage, proviral DNA integration or transcription transactivation (FIG. 8).

Several noteworthy compounds have proceeded through clinical trials[124] including the following compounds. The bicyclam AMD3100 (REF. 125), which specifically targets CXCR4, the co-receptor that is used by T-lymphotropic (or X4) HIV strains to enter cells, and SCH-C (SCH 351125), which specifically targets CCR5, the co-receptor used by macrophage (M)-tropic (or R5) HIV strains to enter cells. New NRTIs such as β-D-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (which is marketed as Reverset™) and amdoxovir (which is also known as DAPD; (−)-β-D-2,6-diaminopurine dioxolane), which are active against HIV isolates with resistance to other NRTIs such as zidovudine and lamivudine. New NNRTIs such as capravirine and etravirine, which are effective against HIV-1 strains carrying the K103N and Y181C mutations that make them resistant to the 'classical' NNRTIs (nevirapine and efavirenz). S-1360, a diketo-acid derivative targeted at the integrase-mediated strand-transfer reaction and the first integrase inhibitor to reach clinical studies. Finally, tipranavir (PNU-140690), which is the first non-peptidomimetic inhibitor of the HIV protease to be brought to the clinic and which is expected to retain activity against HIV-1 isolates that have developed resistance to the 'classical' peptidomimetic PIs.

Several other therapeutic strategies that are still at the preclinical stage are being pursued for the chemotherapy of HIV infections[124]. These include the following strategies. Downmodulation of CD4 — which is the main cellular receptor used by HIV to bind to its target cells — by cyclotriazadisulphonamides (CADAs). The use of compounds that specifically bind to the viral envelope glycoprotein gp120, such as BMS-378806 [4-benzoyl-1-(4-methoxy-1*H*-pyrrolo-(2,3-*b*)pyridin-3-yl)oxoacetyl-2-(*R*)-methylpiperazine], and which thereby block interaction of gp120 with CD4. Using plant lectins derived from snowdrop agglutinin (GNA), amaryllis lectin (HHA) and aglycons of the glycopeptide antibiotics vancomycin, teicoplanin and eremomycin, which specifically interact with gp120. Developing pyranodipyrimidine inhibitors of HIV integrase, such as V-165, that inhibit integration in a manner that is different from the diketo-acid derivatives (such as S-1360) in that they do not show cross-resistance with the diketo-acid derivatives; and finally, two new classes of compounds (*N*-aminoimidazole derivatives (NAIMS) and pyridine oxide derivatives), which seem to inhibit a post-integration, transcription transactivation event, the exact nature of which remains to be elucidated.

Recently, a new class of anti-HIV compounds called maturation inhibitors were identified[126]. PA-457 [3-O-(3′,3′-dimethylsuccinyl)betulinic acid] disrupts a late step in HIV-1 Gag processing that involves conversion of the capsid precursor (p25) to mature capsid protein (p24). Virions released from PA-457-treated cultures are no longer infectious, and resistance mutations have been identified in the p25 to p24 cleavage site[126]. Further studies are required to assess whether PA-456 (and similar compounds) have therapeutic potential for HIV infections.

Conclusion

At present, 37 chemicals, plus IFN-α in both pegylated and unpegylated forms, have been formally approved for the treatment of viral infections, at least half of which are intended to treat HIV infections. A similar number of compounds are also under preclinical or clinical development, at least half of which can be expected to reach the antiviral drug market. Overall, antiviral strategies seem to target the inhibition of viral DNA polymerase for the treatment of DNA virus infections, helicase/NTPase for the treatment of HSV, HCV or SARS-CoV infections, IMP dehydrogenase for the treatment of HCV and some (−)RNA virus (for example, arena- and bunyavirus) infections, SAH hydrolase for the treatment of other (−)RNA virus infections such as Ebola and Marburg virus or (±)RNA virus infections such as rotavirus, and RNA-dependent RNA polymerase for the treatment of other (+)RNA virus (flavivirus and hepacivirus) infections. Finally, IFNs seem to be good therapeutic agents for those viral infections like those caused by coxsackieviruses, hepatitis B and C viruses and SARS-CoV, that, as yet, cannot be sufficiently curbed by other therapeutic or prophylactic approaches.

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Reviews

Hepatitis B virus: old, new and future approaches to antiviral treatment

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Patients chronically infected with hepatitis B virus (HBV) run the risk of developing cirrhosis and hepatocellular carcinoma in later life. Antiviral treatment offers the only means of preventing such an undesirable outcome. To date, interferon-α (IFN-α), an immunomodulator, and two synthetic nucleoside analogues, lamivudine and adefovir dipivoxil, are the only licensed antiviral agents for the treatment of chronic HBV infection. However, the standard treatment endpoints of loss of HBeAg with or without seroconversion to anti-HBe, normalization of serum transaminase levels, loss of HBV-DNA and improvement in liver histology following monotherapy with either types of agent are only achievable in ~20–30% of those treated. Long-term treatment with lamivudine is effective in suppressing viral replication, but drug-resistant mutants arise with increased length of treatment. Nevertheless, such mutants appear to be susceptible to adefovir and other nucleoside analogues that are undergoing Phase II/III clinical trials at the moment. Therapeutic vaccination and other molecular approaches such as antisense oligonucleotides, ribozymes, DNA vaccines, dominant-negative proteins and aptamers are possible future antiviral therapies, which will supplement our armamentarium against chronic HBV infection. It seems certain that combination therapies involving two or more nucleoside analogues, immunomodulators or gene therapies will be the future treatment regimens for chronic HBV infection.

Keywords: HBV, antivirals

Introduction

Conservative estimates place the number of persons chronically infected with hepatitis B virus (HBV) at >350 million.[1] As these patients are at increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC), therapeutic intervention offers the only means of interrupting this progression. The ultimate goals of treatment are to achieve sustained suppression of HBV replication and remission of liver disease. The agents currently available for the treatment of chronic HBV infection are divided into two main groups. The immunomodulators, which include interferon-α (IFN-α), thymosin α1[2,3] and potential therapeutic vaccines,[4] and nucleoside analogues, among which lamivudine (3TC), adefovir dipivoxil, entecavir, emtricitabine, β-L-2-deoxythymidine and famciclovir are the most well-known. At present however, only IFN-α and lamivudine are approved for chronic HBV treatment, and these have recently been joined by adefovir. The immunomodulators act by promoting cytotoxic T cell activity for lysis of infected hepatocytes and by stimulating cytokine production for control of viral replication. Nucleoside analogues on the other hand act by suppressing HBV replication at the level of DNA synthesis, and in addition there is evidence that they may enhance immune clearance of infected hepatocytes.

The hepatitis B virus

Classification

Hepatitis B is the prototype virus of the hepadnaviridae, a name that signifies the hepatotropism and DNA nature of the

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genome of its members. The family includes two genera. The *Orthohepadnavirus* genus contains members that infect mammals, and other than HBV, includes hepadnaviruses that infect woodchucks (woodchuck hepatitis virus, WHV), squirrels, and primates such as chimpanzees, gibbons, gorillas, orang-utans and woolly monkeys.[5] The *Avihepadnavirus* genus contains members that infect birds such as ducks (duck hepatitis B virus, DHBV), herons, storks and geese.[6,7] The WHV and DHBV animal models have proved invaluable in the assessment of the efficacy of potential antiviral agents before human trials, as discussed later on.

Structure

The infectious virion or Dane particle has an outer envelope, which consists of the hepatitis B surface antigen (HBsAg) in a lipid bilayer. This in turn encloses the nucleocapsid core of the virus, within which lies the viral genome. The latter is a relaxed circular, partially double-stranded DNA molecule of 3.2 kb in length, and contains four partially overlapping open reading frames (ORFs) (Figure 1).[8] The Pre-S/S ORF encodes the three envelope glycoproteins that are known as the large (L), middle (M) and small (S) HBsAgs. The precore/core one yields two translation products, the precore polypeptide being the precursor of the soluble hepatitis B e antigen (HBeAg), and the nucleocapsid or core protein. One of the other two ORFs encodes for the X protein and the remaining one for the polymerase, which acts as a reverse transcriptase (rt) and also has DNA polymerase activity.[8,9]

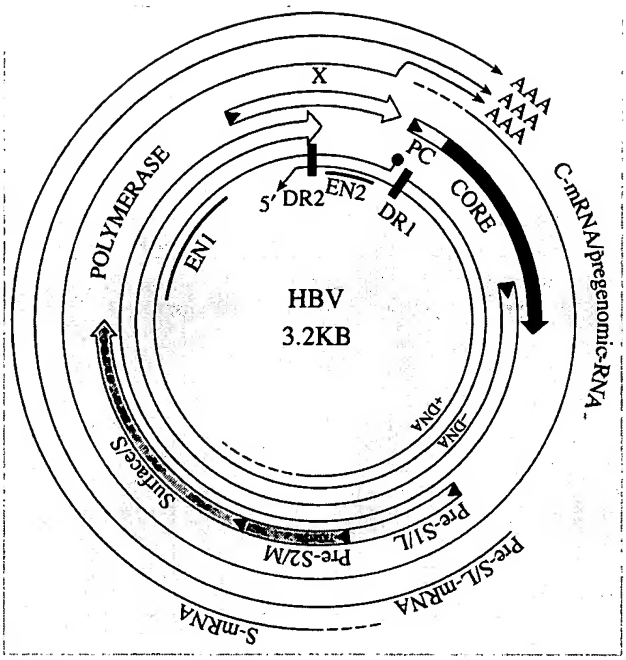

Figure 1. Genomic organization of the hepatitis B virus showing the partially double-stranded DNA and the positions of the direct repeats (DR) 1 and 2, and those of enhancers 1 and 2 (EN). Also shown are the four open reading frames encoding the relevant viral proteins as indicated, as well as the various RNA transcripts terminating at a common polyadenylation signal. Reproduced with permission from The American Society for Microbiology and from Seeger & Mason.[8]

Replication

The life cycle of the virus begins with its attachment to the appropriate hepatocyte receptor, which still remains unknown. In contrast, the region between amino acids 21 and 47 of the Pre-S1 has long been known to be involved in virus binding to the hepatocyte membrane.[10,11] Recently, it has been suggested that a domain within the small S protein may also be involved in attachment to the hepatocyte also, bringing the virus particle into close contact with the cell membrane, and thus facilitating the specific interaction of the Pre-S1 domain with its receptor.[12] The virion is internalized and uncoated in the cytosol, whence the genome translocates to the nucleus, where it is converted into a double-stranded covalently closed circular DNA (cccDNA) molecule, following completion of the shorter positive (+)-strand and repair of the nick in the negative (–)-DNA strand.[8,9,13] In this form, cccDNA serves as the template for viral transcript synthesis by host RNA polymerase II. Most antiviral agents so far have been unable to prevent the replenishment of the cccDNA pool from genomic HBV-DNA recycled from the cytoplasm, or to effect efficient clearance of cccDNA-containing hepatocytes.[14] This explains the rather rapid rebound in serum HBV-DNA after cessation of antiviral treatment.

Viral kinetic studies have indicated that whereas virion half-life is about a day, the half-life of infected cells is much longer and variable, ranging from 10 to 100 days.[15,16] This biphasic response pattern, however, in the case of HBV, and unlike hepatitis C virus (HCV) or human immunodeficiency virus (HIV), may not be universal. Recent findings suggest that viral decay patterns may be more complex or multiphasic (reviewed by Lewin *et al.*[17]), possibly representing both cytolytic and non-cytolytic mechanism involvement in loss of infected hepatocytes. The implications of these findings have a bearing on the development of alternative therapeutic approaches in order to improve the management of HBV-infected individuals. The dosage, duration, timing, combination of antiviral agents and treatment regimens (concurrent, staggered or consecutive) will need to be further optimized if complete eradication of the cccDNA pool is to be achieved. However, this may not be possible in view of calculations that indicate that treatment will be necessary for very long periods of time, making such attempts expensive, impracticable and with an increased risk of breakthrough resistance.

One of the viral RNA transcripts, known as the pregenomic RNA (pgRNA), is longer than genome length (3.5 kb) and forms the template for (–)-DNA strand synthesis, but also constitutes the message for core and polymerase protein translation (Figures 1 and 2).[18] The latter protein has three

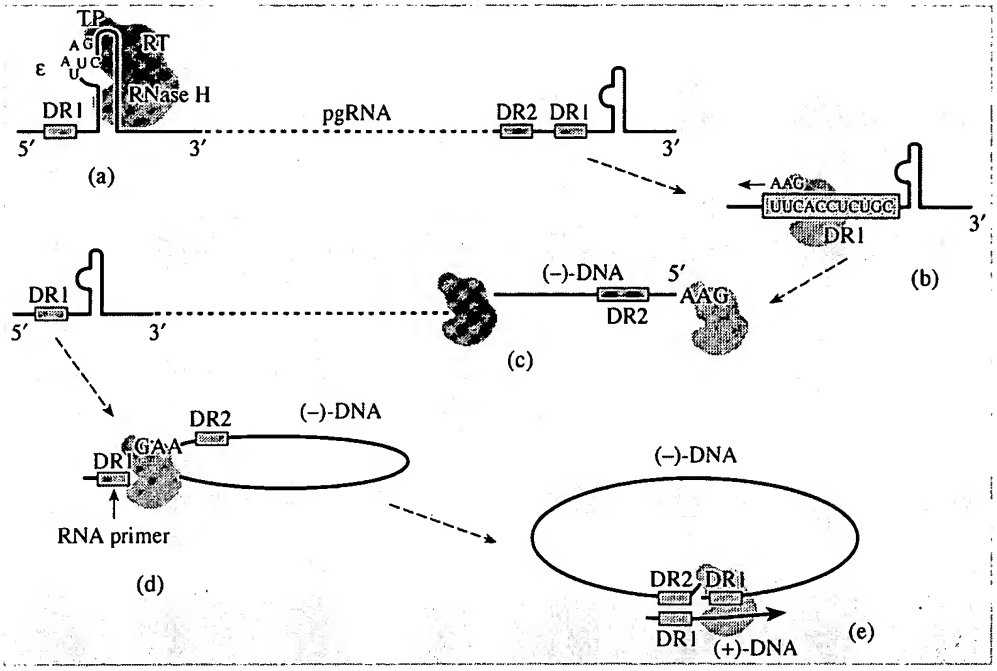

Figure 2. Schematic of the replication mechanism of hepatitis B virus. The viral polymerase binds to ε and directs the synthesis of a short primer using as template the nucleotide sequence of the bulge as shown (a). The polymerase–primer complex translocates to the 3′ end of the pgRNA and base-pairs with DR1, with which is homologous (b). As (–)-DNA strand synthesis proceeds (c), the pgRNA template is degraded by the RNase H domain of the polymerase, apart from its terminal 18 or so bases. These bases constitute the RNA primer which initiates (+)-DNA strand synthesis (d). The primer translocates to the 5′ end of the newly synthesized (–)-DNA strand and anneals with the homologous DR2 region. (+)-DNA strand synthesis then proceeds in the direction shown by the arrow, which necessitates yet another translocation event to the 3′ end of the (–)-DNA strand. Both of these events are most likely to be facilitated by the effective circularization of the (–)-DNA strand. This becomes possible as a result of the covalent attachment of the 5′ end of the strand to the polymerase, which is maintained during continued synthesis of the (–)-DNA strand. Thus, the two ends of the strand are brought into close proximity with each other (e).

functional domains, each in turn involved in DNA priming (terminal protein), reverse transcription and pgRNA degradation (RNase H). There is also a spacer region of unknown function between the terminal protein and the rt domain. Once synthesized, the polymerase engages epsilon (ε), a secondary RNA structure at the 5′ end of the pgRNA,[19] triggering encapsidation of the complex by the core protein. The subsequent steps in virus nucleic acid replication then take place within the nucleocapsid.

The interaction between the polymerase and ε sets in motion the events that take place during viral replication.[8,9,20] As a consequence of the terminal redundancy of the pgRNA, the epsilon sequence and flanking region containing direct repeat 1 (DR1) are duplicated (Figure 2). The bulge of the ε structure serves as a template for the synthesis of a 3–4 nucleotide long DNA primer, which is covalently attached to the polymerase though a tyrosine residue of the terminal protein (position 96).[21,22] This event involves the ε structure at the 5′ end of the pgRNA, and is then followed by the translocation of the polymerase–primer complex to the 3′, where it hybridizes with the DR1 region with which it shares homology. As the complex proceeds towards the 5′ end of the pgRNA, the (–)-DNA strand is synthesized by reverse transcription and the RNA template is concurrently degraded by the RNase H activity of the polymerase, except for the final 18 or so ribonucleotides. A second translocation event then occurs during which the ribonucleotide primer hybridizes with the DR1 region at the 5′ end of the newly synthesized (–)-DNA strand. A template exchange occurs that allows the (+)-DNA strand synthesis to proceed along the 5′ end of the complete (–)-DNA strand, effectively circularizing the genome.[20] (–)- and (+)-DNA strand synthesis occurs within the nucleocapsid as already mentioned, and this is facilitated through pores allowing entry of nucleotides, including nucleoside analogues.[23] Once the maturing nucleocapsid is enveloped by budding through the endoplasmic reticulum membrane,[24] the nucleotide pool within the capsid cannot be replenished, hence the incomplete nature of the (+)-DNA strand.

Mutations

The HBV genome is not as invariant as originally thought. Natural variants of the virus exist, which give rise to well-recognized serological subtypes and its genotypes.[25] However, since HBV replicates through an RNA intermediate that is reverse transcribed, this step in the replication cycle of the

virus is prone to errors. These may occur during pgRNA synthesis by the cellular RNA polymerase II, as RNA polymerases show inherently low copying fidelity, but also during reverse transcription due to the lack of proof-reading capacity by the viral polymerase.[26] Fluctuations in the composition of the intracellular nucleotide pools is another possible contributing factor. Thus HBV has a higher mutation rate than other DNA viruses (2×10^{-4} base substitutions per site per year).[27] Although a lot of these mutations would be deleterious to the virus, as a result of constraints imposed by the overlapping ORFs, some would be advantageous, either offering a replication advantage, or facilitating immune escape. Such are the HBsAg variants[28] and the precore and core-promoter variants.[29-31] The last two variants predominate in anti-HBe-positive patients with detectable levels of HBV-DNA and have important implications in the treatment of such patients with antiviral agents.[32]

The most common precore mutation is the G1896A substitution, which creates a premature termination codon that abrogates HBeAg production.[33] This variant is commonly found in association with HBV genotype D, which prevails in the Mediterranean basin, genotypes B and C, which are prevalent in countries of the far East, and genotype E in Africa. In contrast, this mutation is rarely detected in genotype A strains found in Northern Europe and North America. The selection therefore of the G1896A mutation occurs in patients carrying HBV genotypes with a T at position 1858 in the precore region, which allows for stable base-pairing with the A change at position 1896.[34] The double mutation affecting the core promoter region (A1762T, G1764A) results in decreased transcription of the precore mRNA, with a knock-on effect on HBeAg production, whilst pgRNA production is up-regulated.[35] Patients with HBeAg-negative chronic liver disease tend to have lower HBV-DNA levels than HBeAg-positive patients,[36] and may experience frequent exacerbations with fluctuating transaminase levels.[32,37,38]

Patient groups

HBV is transmitted following perinatal, percutaneous and sexual exposure, but also by contact with open cuts and sores, as may occur between children in hyperendemic areas.[39] Following acute infection, the risk of becoming a chronic carrier of HBV is age dependent. This exceeds 90% in newborns of HBeAg-positive mothers, ranges between 25% and 30% in infants and very young children, but in adults this risk is only between 5% and 10%.[40-42] The patient groups with chronic HBV infection, defined as the persistent presence of HBsAg in the serum of an individual for 6 months or longer,[43] who could benefit from antiviral therapy include:

(i) HBeAg-positive patients who have transaminase levels greater than twice the normal, are positive for HBV-DNA and have necroinflammatory changes in liver biopsy material.

(ii) HBeAg-negative patients, who have active liver disease as shown by transaminase elevations (twice the normal), HBV-DNA positivity ($>10^5$ copies/mL) and moderate to severe hepatitis on biopsy.

(iii) Patients with compensated cirrhosis, and even patients with decompensated cirrhosis with treatments other than IFN.

(iv) Immunosuppressed patients, as a consequence of organ transplantation. Patients immunosuppressed as a result of HIV infection are normally excluded from treatment protocols.

Interferon

Interferons have immunomodulatory, but also antiproliferative and antiviral effects. Lymphoblastoid and recombinant IFN-α, have been used in turn since the early 1980s in attempts to achieve sustained suppression of HBV replication, and remission of HBV-related chronic liver disease. The drug is administered by subcutaneous injection and the recommended dosage for adults is 5 MU (million units) daily or 10 MU thrice weekly for a period of 16 weeks in HBeAg-positive patients, or 12 months for those who are HBeAg-negative. The recommended dose for children is 6 MU/m^2 thrice weekly with a maximum of 10 MU.

Efficacy

A meta-analysis by Wong *et al.*[44] of 15 randomized placebo-controlled studies of IFN-α treatment in HBeAg-positive patients showed loss of HBV-DNA, HBeAg and HBsAg in 37%, 33% and 7.8% of patients, whereas in controls the respective figures were 17%, 12% and 1.8% (Table 1). In Asian patients (primarily Chinese), the treatment is generally less effective, particularly in patients with normal alanine transaminase (ALT) levels.[45] In contrast, those with raised ALT respond similarly to Caucasian patients.[46] This difference in response between Chinese and Caucasian patients is thought to be related to the duration of the chronic state. Whereas in Asian patients, exposure to the virus occurs early in life, either at birth or postnatally, in Caucasian patients infection is acquired during adolescence or adulthood, primarily through sex or intravenous drug abuse. In the former case, infection is followed by a lengthy period of immune tolerance (normal ALT),[47,48] whereas in the latter patients, there is a more active host immune response directed towards clearance of the infection (active liver disease, raised ALT levels).[49] In children, the response rate is similar to that in adults, being ~30% in those with raised ALT, as opposed to 10% in those with normal levels.[47,50,51] The durability of HBeAg loss in the above patient groups is as high as 90%, after many years of follow-up, whereas HBsAg clearance during this time is more varied, ranging from 12% to 65%.[52-58]

Criteria for determining IFN-α treatment efficacy in HBeAg-negative patients include serum HBV-DNA nega-

Table 1. Antiviral responses following therapy with either IFN-α or lamivudine in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B. The figures given are those at the end of the follow-up period[49,59,112]

	Interferon		Lamivudine	
	patients	controls	patients	controls
HBeAg-positive				
loss of HBV-DNA	37%	17%		
loss of HBeAg	33%	12%	17–33%	11%
HBeAg seroconversion	18%[a]		16–18%	5%
loss of HBsAg	7.8%	1.2%	<1%	0
HBeAg negative				
loss of HBV-DNA	28%	10%	25–30%	–
HBV-DNA –ve/ALT normal	18–25%	0	11–20%	–
loss of HBsAg	2.5%	0	0	–

–, Data not available.
[a]Difference in proportions.

tivity and normalization of ALT. Response rates have been variable as a result of heterogeneity in disease patterns (continuous activity, fluctuating or intermittent), virus variation (genotypes, precore variants, basic core promoter variants), treatment regimens and their duration. Most of the data available originate from Greece and Italy,[59–67] countries where HBV genotype D is prevalent and the precore stop codon mutation is common. Nevertheless, end of treatment response rates range between 38% and 90%, which, however, are not sustainable, as virological relapses are quite common, ranging from 54% to 87% (sustained response 18–25%). Similar rates of response (18–23%) have been reported in relapsed patients who have been retreated with IFN.[68,69] About a third of the sustained responders may seroconvert to anti-HBs also.[59,65,67,68] More importantly, IFN-α treatment has been shown to slow down disease progression in comparison with untreated controls, improve survival and reduce HCC occurrence.[64,65]

In decompensated HBV-related cirrhosis, IFN-α administration is not recommended even though it could be of benefit, particularly in Child's A cirrhotic patients, since it is frequently associated with major complications such as variceal bleeding, aggravation of ascites or encephalopathy, development of pneumonia, bacterial infections and gastric ulcer bleeding.[70,71] However, such severe side-effects were shown to be relatively uncommon following prolonged treatment (up to 48 months) with low doses of IFN-α (3 MU three times a week) and careful monitoring of the patients. Sustained loss of serum HBV-DNA and HBeAg, with normalization of ALT and clinical improvement with good survival rate, were observed in 65% of patients.[72]

Patients with decompensated cirrhosis are potential candidates for orthotopic liver transplantation. Such patients with active viral replication have a high rate of HBV recurrence and reduced survival post-transplant due to accelerated HBV-related allograft disease, and this, in spite of passive immunoprophylaxis with anti-HBs (hepatitis B immune globulin; HBIg) in the immediate post-transplant period. Interferon can therefore be administered pre-transplant to reduce viral load, which together with HBIg post-transplant would prevent reinfection of the new liver graft. This has been shown to be the case in two studies using 1–3 MU three times a week of IFN-α.[73,74] In both studies, attainment of HBV-DNA negativity before transplant was essential in preventing re-infection.

The efficacy of other more recently developed interferons, such as consensus and pegylated interferon, which have been used in the treatment of chronic HCV infection, is presently being assessed in clinical trials in HBV carriers.

Predictive factors

Factors that have been associated with a favourable outcome following IFN-α treatment include high pre-treatment ALT and low serum HBV-DNA levels,[75–77] parameters that indicate that the patient is already in the immune clearance phase. In contrast, factors that have been associated with poor response include male sex, length of chronic state, Asian origin, precore mutations, homosexuality and HIV co-infection. HBV genotype in relation to IFN-α response rates has not been investigated in any of the clinical trials so far. In a recent retrospective study from Taiwan, however, it was established that patients with genotype B were more likely to respond to IFN treatment than those with genotype C (41% versus 15%).[78] The latter patients had higher ALT levels and a higher frequency of core promoter mutations.

Prednisolone priming

Early observations suggested that steroid withdrawal is associated with an immunological rebound resulting in HBV-DNA decline. This might be beneficial to the patient if it is timed to coincide with the start of IFN-α therapy. A meta-analysis however of several clinical trials of IFN-α use after priming with prednisolone showed that such an approach was of marginal benefit and in only some of the patients.[79,80] This contrasts with the findings of a European study reporting that prednisolone priming resulted in a higher rate of HBeAg seroconversion than IFN alone.[81] A major drawback in this approach is the danger of hepatic decompensation following steroid withdrawal, an undesirable event that has led to the abandonment of this type of treatment.

Adverse effects

IFN-α therapy is associated with a number of adverse effects. Among these, flu-like symptoms that can be relieved with paracetamol, fatigue, leucopoenia and depression are the most frequently reported. Hair loss, anorexia, mood swings and irritability have also been reported. Finally treatment with IFN may unmask or aggravate underlying autoimmune disorders, such as thyroiditis.[82]

Other immunomodulators

It is generally accepted that patients with chronic HBV infection have weak and restricted T cell responses, whereas such responses are robust and multispecific during recovery from acute infection.[83] Therefore, immunomodulators that can stimulate T cell responses may be effective in the treatment of chronic HBV patients. Thymosin-α1 is a synthetic peptide of 28 amino acids, which promotes T cell maturation and induces cytokine production, including IFN-γ and interleukin 2 (IL-2). Early experiments with thymosin in chronically infected woodchucks showed a 1000-fold reduction in serum WHV-DNA levels in four of the six animals that were treated.[84] In human trials, thymosin was well tolerated, but data on efficacy gave somewhat mixed results. Two trials, one in Chinese patients and the other in patients from the USA, reported loss of HBeAg and HBV-DNA in 41% and 27% of patients treated for 6 and 12 months, respectively, versus 9% of controls in the former trial,[3] and 14% versus 4% after 6 months of therapy in the second.[85] Evaluation was at 18 and 12 months, respectively, after start of treatment. A recent meta-analysis of five trials indicated that thymosin is effective in suppressing viral replication in chronic HBV infection, but this effect is delayed and becomes apparent 12 months after the end of treatment.[86]

Cytokines other than the IFN-α and -β have also been used in the treatment of chronic HBV infection. Interleukin-12 (IL-12), which favours the differentiation of T helper cells to Th1 cells, was evaluated in a pilot study during which 46 HBeAg-positive patients were treated for 12 weeks with different doses of a recombinant human IL-12 preparation. After a further 12 weeks of follow-up, seroconversions were seen only in the higher dose groups, which amounted to a modest 16%.[87] More studies are therefore needed to establish whether IL-12 has any role in the treatment of chronic HBV. The efficacy of other cytokines such as IL-2 and IFN-γ, and immunostimulants like levamisole, in the treatment of chronic HBV patients has been disappointingly poor.

Nucleoside analogues

How they work

Nucleoside analogues are chemically synthesized drugs that are able to mimic natural nucleosides. As such, they are incorporated into newly synthesized HBV-DNA causing chain termination, and thus inhibiting viral replication. In addition, some of them competitively inhibit the DNA-dependent and reverse transcriptase activity of the viral polymerase. For this to occur, the analogues need to be phosphorylated within cells to their triphosphate counterparts. Nucleoside analogues can be produced in their natural D- or unnatural L-configuration, and these are often referred to as enantiomers. Template-dependent DNA polymerases add both L- and D-enantiomers of dNTP analogues to DNA with equal efficiency, when there is no 3′ substituent present.[88,89] Interestingly, however, the HBV polymerase has a preference for the L- over the D-configuration enantiomers.[90] L-Configuration enantiomers appear to have antiviral activity comparable to and sometimes greater than that of their D-counterparts, are less toxic and have greater metabolic stability. Steps in the life cycle of HBV that may be inhibited by nucleoside analogues include the synthesis of the (–)-DNA strand by reverse transcription, the synthesis of the (+)-DNA strand, the amplification and replenishment of the cccDNA pool in the hepatocyte nuclei from non-enveloped core particles, and the cccDNA formation in newly infected cells. Experiments in woodchucks suggest that nucleoside analogue treatment does not have an appreciable effect on the cccDNA pool in hepatocytes.[91] Similar studies in a recombinant baculovirus–HepG2 cell system showed inhibition of cccDNA accumulation, only if lamivudine was present before infection.[92] More recently, it was observed that cccDNA declined exponentially in DHBV congenitally infected ducks on combination therapy with lamivudine and a dideoxyguanosine prodrug. This decline was seen in animals whose liver biopsies had significantly greater numbers of nuclei staining positive for the cell division marker PCNA, than in animals in which cccDNA levels had reached a plateau.[93] The effectiveness of nucleoside ana-

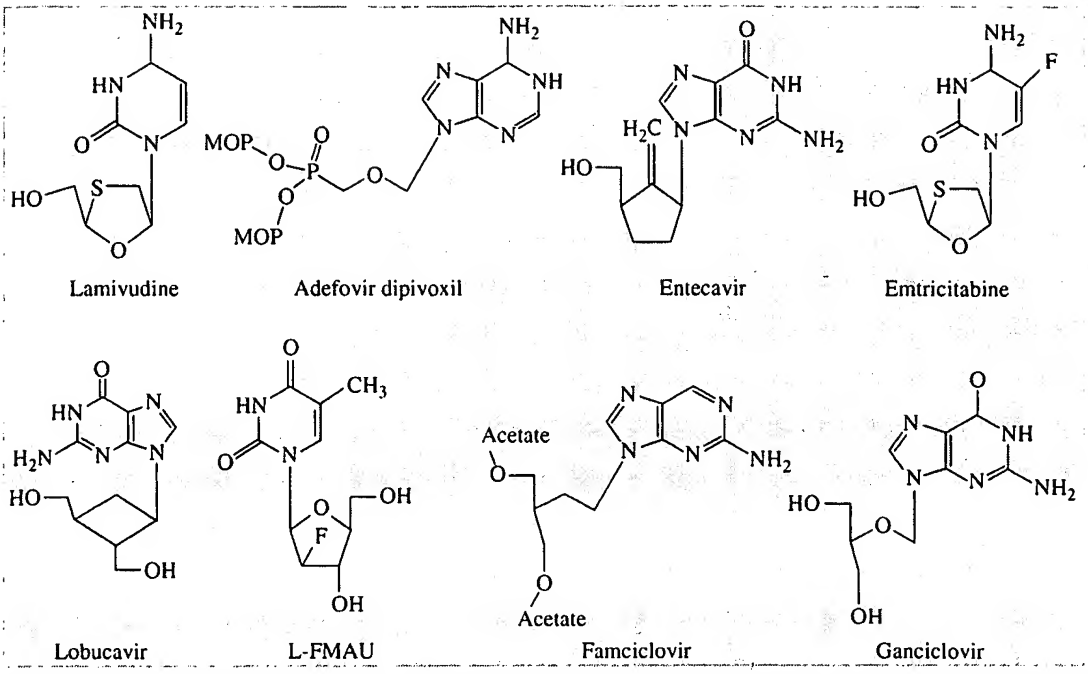

Figure 3. Chemical structure of some of the nucleoside analogues that are presently used, or have been used in the past, for the treatment of chronic HBV infection. POM, pivaloyloxymethyl.

logues in reducing the cccDNA pool may thus be dependent on the cell cycle phase.

Lamivudine

Lamivudine, also known as 3TC (Epivir) is the L-enantiomer of the deoxycytidine analogue 2′,3′-dideoxy-3′-thiacytidine. This is metabolized within hepatocytes to the active triphosphate, by stepwise addition of phosphate groups (Figure 3).[94,95] The drug contains a sulphur atom instead of carbon at the 3′ position of the sugar ring, which does not allow chain elongation by phosphodiester bond formation, in the absence of the normal 3′ hydroxyl group. Since lamivudine acts by terminating viral DNA synthesis[95,96] and competitively inhibiting the viral polymerase/rt,[96] it is equally effective in patients of any race, but also against both the wild-type virus and precore/core promoter variants.[97–101] In addition, there is evidence to suggest that lamivudine treatment may overcome cytotoxic T cell hyporesponsiveness seen in chronically infected patients.[102]

Dose

Lamivudine is administered orally and the recommended dose for adults is 100 mg per day, whereas that for children is 1 mg/kg per day rising to 100 mg/day. The duration of treatment is 1 year in HBeAg-negative, and can be longer than a year in anti-HBe-positive patients.

Efficacy

Several randomized clinical trials in HBeAg-positive patients showed that a 1 year course of lamivudine monotherapy induced HBeAg seroconversion in 16–18% of them, compared with 4–6% in controls.[103–105] Histological improvement by at least two points in the histological activity score was observed in 49–56% of those treated and in ~25% of controls. Subsequent studies showed that HBeAg seroconversion rates increased with length of therapy rising from 17% at 1 year to 27%, 36% and 47% at years 2, 3 and 4, respectively.[106–108] Despite the emergence of lamivudine-resistant variants, which are described further on, continued treatment results in further HBeAg seroconversions, and median HBV-DNA and ALT levels are maintained at lower than baseline values.[106,107] The most important predictor of a favourable response following lamivudine treatment is the pre-treatment ALT level.[109] It has been shown that the higher the ALT, the higher the rate of HBeAg seroconversion, rising from 2% in those with normal ALT to 47% in those with levels five times the upper limit of normal.[110]

The efficacy and tolerability of lamivudine as a treatment for chronic HBV infection in children have also been investigated. In a recent trial, the rate of HBeAg loss and HBV-DNA negativity was higher among 191 children who received lamivudine than among the 97 who received placebo, at week 52 of treatment (23% versus 13%, $P = 0.04$).[111]

Treatment of HBeAg-negative patients with lamivudine results in response rates that are equivalent to those in HBeAg-

positive ones. The results from several studies have recently been reviewed by Rizzetto[112] and indicate that end points of loss of HBV-DNA and ALT normalization were achieved in 65–96% of patients at the end of treatment,[97,98,113–118] but patient relapse rates after 1 year of follow-up amounted to 45–74% (sustained response of 11–20%). Histological improvement as demonstrated by a reduction in the Knodell necroinflammatory score was seen in 60% of patients.[97] In the same study, the fibrosis score improved in 11%, and remained the same in 86% of patients. Two other studies reported histological improvements of 22%[118] and 95%,[119] and in addition, 35% of patients had improvement in fibrosis in the latter study. It appears therefore that lamivudine treatment results in histological improvement, which is comparable between HBeAg-positive and -negative patients. Moreover, the arrest or reduction in the immune-mediated inflammatory response in the liver leads to reduced scarring, with a consequent beneficial effect on fibrosis.[120] Finally, lamivudine treatment has been used in patients with severe acute exacerbations complicating chronic HBV infection, and shown to be effective, even in patients with hepatic failure, 8/10 of whom had uneventful recoveries.[121]

The efficacy of lamivudine in HBeAg-negative patients has also been evaluated for periods >12 months. Response rates as defined above generally dropped by 24 months of treatment, by between 14% and 36% of those at 12 months.[98,114,115,122] For example, virological response rates of 68% at 12 months decreased to 52% and 41.6% at 18 and 24 months, respectively.[114] Such breakthroughs are invariably due to HBV variants with mutations in the polymerase gene and these are discussed in detail later on. Other than the duration of treatment, core promoter mutations have been associated with the selection of lamivudine-resistant mutants.[98]

Lamivudine treatment of patients with decompensated cirrhosis has shown that the drug is well tolerated and leads to improvement in the clinical picture of many patients, to the extent that some of them can be removed from liver transplantation lists.[123–126] In the liver transplantation setting, treatment with lamivudine inhibits HBV replication and improves liver function in patients awaiting a liver transplant.[127–131] HBV-DNA levels become undetectable after just 12 weeks of treatment,[128,130] whereas histological improvement in the necroinflammatory and fibrosis scores was seen in 50% and 26% of patients, respectively.[129,131] In a recent trial where lamivudine was used before and after transplantation, 60% (25/42) of patients were HBsAg-negative at their last visit (12 weeks or more after transplantation). At week 156, 59% (13/22) still remained HBsAg-negative. All of the re-infected patients (nine in all) were HBV-DNA-positive before transplantation.[132] Prevention of recurrence of infection after liver transplantation has also been attempted with lamivudine in combination with HBIg.[133] In this study, lamivudine treatment was initiated before or at the time of transplantation, and continued thereafter. At 1 year, actuarial patient and graft survival was 93%, and at a median 346 days after transplantation, all surviving patients (13/14) had undetectable HBV-DNA. In another study, low risk liver transplant patients (HBsAg-positive/HBV-DNA-negative) on HBIg treatment for 6 months after transplantation were randomized to receive either lamivudine or continue on HBIg; 11/12 patients on HBIg and 10/12 on lamivudine remained HBsAg-negative, but HBV-DNA was detectable by PCR in 6/8 patients on HBIg and 5/7 on lamivudine over a period of 6–22 months. All patients remained HBsAg-negative with normal graft function.[134] Development of anti-HBs with prolonged lamivudine treatment has also been reported.[135]

Finally, prednisolone withdrawal followed by lamivudine treatment gave virological responses in 60% of patients with ALT over five times the upper limit of normal, and only in 10% of those with no significant ALT rebound.[136]

Long-term outcome

The durability of HBeAg seroconversion in lamivudine-treated patients is variable, ranging in some studies between 38% and 73%,[107,108,137] whereas in others it was maintained.[103,138] In one study, 9% of patients lost HBsAg also, after a median follow-up of 21 months.[139] Post-treatment responses have also been evaluated in HBeAg-negative patients for up to a period of 2 years. A sustained virological and biochemical response was maintained in only 11–20% of patients.[97,113,118] Relapse rates were 48% after 6 months of follow-up and rose to 74% by 12 months.[113]

Drug resistance

Of major concern is the emergence of drug-resistant variants of HBV following lamivudine treatment. Breakthrough infections, indicated by virological and biochemical relapses, have been recorded in 14–32% of HBeAg-positive patients treated with lamivudine for a year.[103–105] With longer periods of treatment, resistance was shown to increase from 14% at 1 year to 38%, 49% and 66% for years 2, 3 and 4, respectively.[106–108] Lamivudine-resistant variants also arise in HBeAg-negative patients, but rates are more variable, ranging from 0% to 27% at 1 year to between 10% and 56% at 2 years.[97,98,113,115] Emergence of the lamivudine-resistant variants may be accompanied by acute exacerbation of liver disease,[140,141] and although rare, there have been isolated cases of hepatic decompensation. Continuation of treatment in patients with lamivudine-resistant variants sustains serum HBV-DNA and ALT at levels lower than those at the start of therapy. Moreover, HBeAg seroconversion has been reported to occur in about a quarter of the patients with breakthroughs who continue treatment.[106,140] It has been observed that if after 24 weeks of lamivudine treatment the patient is still HBeAg/HBV-DNA-positive and the ALT is higher than 1.3× the

upper limit of normal, then there is a 99% likelihood that polymerase mutants have arisen.[142]

As mentioned previously, the polymerase is divided into four domains, one of which functions as the rt of the virus. This region contains at least five subdomains (A–E), which are spatially separated but closely associated with the normal function of the protein. Similar to other rts,[143,144] the HBV polymerase is thought to assume a three-dimensional, right-handed conformation, consisting of thumb, palm and finger domains. The latter contains subdomains A, C and D, which are most likely to be involved with dNTP binding and catalysis, whereas subdomains B and E interact with the pgRNA template and primer, and correspond to the thumb and palm of the structure, respectively.[145,146] The HBV polymerase, as other RNA-dependent polymerases, contains the characteristic YMDD (tyrosine-methionine-aspartate-aspartate) motif of the catalytic site, located within subdomain C.[147] The most common amino acid substitutions that have been described and are associated with lamivudine resistance occur in both the B and C subdomains, and have been clearly shown to confer drug resistance.[141,148,149] These arise as a result of point mutations in the nucleotide sequence, affecting the relevant codons. It should be noted that not all point mutations result in amino acid changes, this being due to the degeneracy of the genetic code. Amino acid substitutions that confer drug resistance predominantly affect the YMDD motif, so that the methionine (M) at position 552 is changed either to valine (YVDD) or isoleucine (YIDD).[104,141,148,150–154] The former mutation is almost always associated with a second one in subdomain B, a substitution of leucine with methionine at position 528 (L528M).[148,151,153,154] A new numbering system by which changes in the amino acid sequence of the polymerase protein are identified has recently been proposed.[155] In this system, the amino acids of each polymerase domain are numbered separately, so that the aforementioned rt domain mutations become rtM204V/I and rtL180M, respectively. In one study, about one-third of patients had the rtM204I mutation and the rest the rtL180M/M204V change.[140] Other mutations that have been described include the rtV173L (V521L) and rtF166L (F514L), both in domain B (Table 2).[141,156,157] One other mutant, rtA181T (A529T), has been shown to be resistant to lamivudine following prolonged treatment.[158] The same study reported replacement of the original YMDD mutants with distinct ones during prolonged treatment. More recently, an rtM204S (M552S) mutant has been described, with the accompanying rtL180M (L528M) change.[159]

Crystallographic data have shed further light on the mechanism of lamivudine resistance. The YMDD mutations seem to affect the ability of the dNTP-binding pocket to accommodate the drug. This in turn leads to a reduction in the affinity of lamivudine for the rt domain, and possibly those of the natural nucleotides also.[145,146,148,160,161] Moreover, the amino acid changes may alter their precise spatial arrangement necessary for optimum function during catalysis. Thus, the low affinity occupancy of the site is further compounded by suboptimal catalytic efficiency.[145,146] The rtL180M mutation, which is spatially very close to the rtM204 position, may represent an attempt by the virus to partly redress this problem. This may also explain the observation that lamivudine-resistant variants are less replication fit than the wild-type

Table 2. Amino acid mutations in the rt domain of the viral polymerase commonly associated with resistance to lamivudine and famciclovir. These are presented in both the old and new amino acid numbering systems[155,156,157,186–189]

	Subdomain B		Subdomain C	
Numbering system	old	new	old	new
Amino acid position	511–537[a]	163–189[b]	545–558	220–210
Lamivudine	L528M	rtL180M	M552V/I	rtM204V/I
	V521L	rtV173L	M552S	rtM204S
	F514L	rtF166L	V555I	rtV207I
	A529T	rtA181T		
	T532S	rtT184S		
Famciclovir	L528M	rtL180M	V555I/E	rtV207I/E
	V521L	rtV173L		
	P525L	rtP177L		
	T532S	rtT184S		
	R501Q	rtR153Q		
	A529V	rtA181V		

[a]Based on genotype A.
[b]Standardized numbering for all genotypes.[155]

virus,[162,163] whereas the rtL180M mutation has no impact on HBV replication on its own.[163] Ono *et al.*[163] established that the rtL180M mutation in combination with the rtM204V change partly restored the replication competency of the C domain mutants, and in addition increased resistance to nucleoside analogues. The reduced replication capability of the resistant variants may explain the rapid re-emergence and take-over by the wild-type virus once treatment is stopped.[150] This in addition, confirms the failure of the drug to eliminate cccDNA-containing hepatocytes. To avoid the re-emergence of the wild-type virus and a possible rebound in ALT levels, it is recommended that patients with breakthrough infection are maintained on lamivudine treatment long-term. A recent study, however, suggested that in the liver transplantation setting, lamivudine treatment may result in the selection of polymerase mutants with increased levels of replication, confirmed *in vitro* in the presence of the drug. These were associated with mutations in the YMDD motif, and in addition in the 'a' determinant of the overlapping surface gene, which may represent compensatory changes to restore replication competency.[164,165]

In vitro studies using transient transfection cell culture systems have confirmed that the above YMDD mutations confer lamivudine resistance. Moreover, such systems have been very useful in establishing sensitivity, or cross-resistance, to other nucleoside analogues. Lamivudine-resistant full-length HBV-DNAs containing the rtM204I, M204V or L180M/M204V mutations have been used to transfect human hepatoma cells. These experiments showed that lamivudine had no effect on the replication of the mutant viruses, whereas adefovir dipivoxil and lobucavir {9-[1β-2α-3β]-2,3-Bis-(hydroxymethyl)cyclobutyl]guanine} were active against all mutants,[163,166–168] as well as DAPD [(–)-β-D-2,6-diamino-purine dioxolane][169] and DXG, its active metabolite following deamination.[170] The rtM204V mutant appeared to be sensitive to L-FMAU[163,169,171] and tenofovir.[171] However, in a recent study, lamivudine-resistant mutants were shown to be resistant to L-FMAU also.[172] Resistance was also maintained against drugs such as penciclovir, emtricitabine and others.[163,169,171]

Other nucleoside analogues

A number of other nucleoside analogues have been tested or are being evaluated presently against HBV in Phase II and III clinical studies.

Famciclovir

This is the oral prodrug of penciclovir {9-[4-hydroxy-3-(hydroxymethyl)butyl] guanine}, an acyclic deoxyguanosine analogue (Figure 3). Famciclovir is deacetylated and oxidized to penciclovir, which is in turn phosphorylated in hepatocytes to the triphosphate by cellular enzymes. This then competes with dGTP as a substrate for the HBV polymerase. The drug is incorporated into nascent HBV-DNA strands causing premature termination,[173,174] but it can also interfere with the priming of reverse transcription, by binding to the tyrosine residue of the terminal protein, which is involved in primer synthesis.[175]

Penciclovir has been shown to inhibit viral replication in Pekin ducks infected with DHBV.[176–178] However, after drug withdrawal there was a rebound of viral markers to pre-treatment levels.[176] This again is thought to be due to the persistence of cccDNA in hepatocytes. In clinical studies famciclovir has been found to be well tolerated and to inhibit HBV replication.[179,180] A 500 mg dose thrice daily has been shown to give the best results, but the length of treatment in various studies has been variable. A pilot study showed >90% reduction in HBV-DNA levels in ~55% of patients treated for 10 days.[179] A larger multicentre placebo-controlled trial showed that the 500 mg three times a day for 16 weeks, apart from inhibiting virus replication, was associated with sustained normalization of ALT and gave anti-HBe seroconversion in 14% of patients, by the end of the 8 month follow-up period.[180] In another study, HBeAg seroconversion following famciclovir treatment was more modest at 9% compared with 3% in the control group.[181]

Famciclovir has also been used in patients with decompensated cirrhosis and shown to improve liver function; 50% of patients given 500 mg three times a day became HBV-DNA-negative after 44 days of treatment.[182] In the liver transplantation setting, patients treated with famciclovir had greatly reduced HBV-DNA levels, accompanied by histological improvement.[183] In larger clinical trials, where patients with recurrent HBV after orthotopic liver transplantation were treated with famciclovir long-term, median HBV-DNA level reductions of 91%, 95% and 99% were seen at 6 months, and at 1 and 2 years, respectively. The median reduction in ALT levels was 58% and 90% at the yearly time points.[184,185] Data on the efficacy of famciclovir in HBeAg-negative patients are rather lacking.

Prolonged treatment with famciclovir results, as in the case of lamivudine, in drug resistance. A number of mutations have been described affecting almost exclusively amino acids in the B domain of the polymerase [rtL180M, V173L, P177L (P525L), T184S (T532S), R153Q (R501Q), in domain B, and rtV207I (V555I) in domain C] (Table 2),[156,157,186–189] although mutations at other positions have also been described, including intersubdomain regions and the terminal protein.[187] The rtL180M mutation is however the dominant one seen in patients with breakthrough infections whilst on famciclovir treatment, and this is a risk factor in patients treated with lamivudine subsequently. Resistance to lamivudine occurs earlier in such patients who are sequentially treated with famciclovir followed by lamivudine.[157]

In *in vitro* studies, the rtV207I mutation exhibited the highest resistance to penciclovir, whereas the rtV173L and L180M mutations showed moderate resistance.[190] All the famciclovir mutations in cross-resistance studies were shown to be sensitive to adefovir, whereas rtV173L, L180M and T184S were also sensitive to lamivudine. The remaining ones displayed moderately decreased sensitivity to lamivudine.

In view of its low efficacy, high dosage and potential for cross-resistance with lamivudine, famciclovir has not become one of the established treatments for chronic HBV. However, it has potential if used in combination therapies and this is discussed later.

Adefovir dipivoxil

Adefovir (Hepsera) or bis-pivaloyloxymethyl-9-(2-phosphonyl-methoxyethyl) adenine (PMEA) is a phosphonate of an acyclic nucleotide analogue (Figure 3). The drug, unlike other nucleoside analogues, contains a phosphate group already and requires an additional phosphorylation step (diphosphate), before it becomes active. This is preceded by the removal of the bis-pivaloyloxymethyl moiety. Adefovir, other than acting as a DNA chain terminator, is also thought to stimulate natural killer cell activity and to induce endogenous interferon production.[191] Adefovir is a potent inhibitor of HBV replication,[192,193] and has been assessed for efficacy in the clinical setting.[167,193–196] Treatment with adefovir results in a rapid decline in HBV-DNA levels within 2 weeks.[167,197] The drug is active against lamivudine-resistant mutants,[166,167,198–200] and no resistance to it has been seen in patients treated with the drug for 48–60 weeks.[198,201] At doses of 60–120 mg daily, as used in HIV-infected patients, the drug was shown to have mild to severe nephrotoxic effects.[202] In the HBV trials a 10 mg daily dose was used, and no such side-effects have been recorded. Experiments in the woodchuck and duck hepadnaviral animal models have shown that adefovir, similarly to other nucleoside analogues, does not eradicate the hepatocyte cccDNA pool.[203,204]

Adefovir appears to have potential as an effective drug against HBV, and may prove a strong candidate in combination therapies. The drug under the trade name Hepsera received approval by the FDA for use in the treatment of chronic HBV infection, in September of 2002.

Entecavir

Entecavir is a carbocyclic 2′-deoxyguanosine analogue with potent anti-hepadnaviral activity {1s-(1α,3d,4β)-2-amino-1,9-dihydro-9-[4-hydroxy-3-(hydroxymethyl)-2-methylene-cyclopentyl]-6H-purin-6-one monohydrate} (Figure 3).[205–207] In chronically infected woodchucks, treatment with entecavir resulted in a 2–3 $\log_{10}$ reduction in serum WHV-DNA, which rebounded on stopping treatment.[206,207] Similar results were obtained with the duck animal model.[208] Entecavir was shown to be well tolerated, with no evidence of resistant mutants in two groups of woodchucks maintained on the drug for 14 and 36 months, respectively;[209] 50% of the animals from the first group (withdrawn from therapy) had a sustained antiviral response 28 months later, whereas in the group treated longer, two-thirds of the animals had DNA levels near the lower limit of detection for 2 years. There was no emergence of HCC in either group. In a recent trial, entecavir was shown to be superior to lamivudine in reducing serum HBV-DNA in patients with chronic HBV infection.[210] Entecavir has also been shown to suppress lamivudine-resistant mutants *in vitro*.[211] In humans, a randomized, placebo-controlled, dose escalating study in patients with chronic HBV infection examined the safety and efficacy of the drug given for 28 days. HBV-DNA was suppressed by all four doses used, more so with the 0.5 and 1 mg once-daily doses (mean 2.8 and 2.5 $\log_{10}$ reduction, respectively).[212] After 24 weeks of follow-up, HBV-DNA returned, but at a slower rate in the higher doses of the drug.

Emtricitabine (Coviracil)

This is a 5-fluorinated derivative of lamivudine, [(–)-β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine] or [(–)FTC], which is converted to the triphosphate by cellular enzymes and competes with dCTP as a substrate for HBV polymerase (Figure 3).[213] (–)FTC was found to be a potent inhibitor of HBV replication in the human hepatoblastoma cell line 2.2.1.5,[213] in primary human hepatocytes,[215] and *in vivo* in nude mice.[216] In the woodchuck animal model (–)-FTC reduced WHV-DNA significantly in a dose-dependent manner, showing antiviral activity levels similar to those obtained with lamivudine.[217,218] In a dose range study in chronic HBV carriers, daily doses of 100 mg or greater suppressed HBV-DNA levels by between 1.7 and 3.3 $\log_{10}$ over the 2 month dosing period.[219] However, cross-resistance between lamivudine and FTC has been reported, thus precluding its use in the treatment of patients with lamivudine-resistant variants.[220,221]

Ganciclovir

Ganciclovir or 2-amino-1,9-{[2-hydroxy-1(hydroxymethyl)-ethoxy]methyl}-6H-purin-6-one is a deoxyguanosine analogue (Figure 3), and has potent antiviral activity against DHBV. Ganciclovir has been shown to suppress viral replication and cause histological improvement, following recurrent HBV infection in liver transplant patients.[222,223] In patients with chronic HBV infection, ganciclovir suppressed HBV-DNA levels by 99%, and this marker became negative in 26%, whereas ALT normalized or declined in most patients. However, there was a rebound in HBV-DNA levels on stopping therapy during the 8 week follow-up period in

60% of patients.[224] Oral administration of the drug was very well tolerated. However, the drug has frequent and potentially serious adverse effects, which are likely to limit its long-term use in the treatment of chronic HBV infection.

In the duck animal model, ganciclovir has shown potent antiviral acitivity.[225] However, treatment of ducks with ganciclovir for 24 weeks did not have substantial impact on the cccDNA pool or viral RNA levels, and gave an increase in hepatic expression of envelope proteins.[226]

FIAU

This nucleoside analogue [-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-iodo)-uracil], or fialuridine in short, was used in a Phase II clinical trial, which was stopped prematurely due to serious clinical side effects. In *in vitro* studies, FIAU was shown to be effective in suppressing HBV replication.[227] However, FIAU was found to have a high affinity for polymerase γ, which incorporates the drug into mitochondrial DNA.[228] As a result, when it was used *in vivo*, several patients developed severe liver and kidney dysfunction associated with lactic acidosis, which led to some fatalities.[229,230] This highlights the importance of having full *in vitro* toxicology data and complete evaluation of the drug affinities for cellular polymerases, well before use in human trials.

Others

Other nucleoside analogues that have been developed and tested both *in vitro* and some cases *in vivo* include: lobucavir,[231] which has been suspended due to possible drug-related increase in tumour incidence in rodents; L-FddC (2′,3′-dideoxy-β-L-5-fluorocytidine),[232] L-FMAU or clevudine 1-(2′-fluoro-5-methyl-β-L-arabinofuranosyl)-uracil,[233,234] DAPD and DXG mentioned earlier,[169,170] β-L-Fd4c (β-L-2′,3′-dideoxy-2′3′-didehydro-5-fluorocytidine)[235] and MCC-478.[236,237] Three additional simple and related nucleosides, β-L-2′-deoxycytidine (LdC), β-L-thymidine (LdT, telbivudine) and β-L-2′-deoxyadenosine (LdA), have been discovered to be potent, specific and selective inhibitors of HBV, WHV and DHBV replication.[238–240] Finally, tenofovir disoproxil fumarate, an acyclic nucleoside analogue closely related to adefovir, which is directly incorporated into DNA, has recently been shown to cause significant (4 $\log_{10}$) reduction in serum HBV-DNA and seroconversion to anti-HBe in 25% of patients treated for 1 year.[241] Moreover, it was active against lamivudine-resistant strains.[242,243]

Combination therapy

In view of the shortcomings of antiviral monotherapy (restricted efficacy, drug resistance), combination therapy with a number of nucleoside analogues, with or without IFN-α, may result in greater sustained response rates.

Combination of IFN-α and lamivudine

Combination therapy with IFN-α and lamivudine was shown in one study to result in higher HBeAg seroconversion rates (29%) than either drug alone (18%), which did not reach significance.[105] In this study, combination therapy was initiated after 8 weeks of lamivudine treatment, and continued for 16 weeks. Similar results were obtained following combination treatment for 24 weeks.[244] HBeAg seroconversion and HBV-DNA negativity was seen in 33% of combination-treated patients as opposed to 15% of those treated with lamivudine alone. In addition, histological improvement was seen in 46% and 27% of patients, respectively. Lamivudine monotherapy in both studies was for 52 weeks. Treatment of chronically infected woodchucks with a staggered regimen involving lamivudine alone for 12 weeks, combination of lamivudine and recombinant human IFN-α for a further 12 weeks, and then 12 weeks of IFN alone, was shown to be better at suppressing WHV replication than either monotherapy alone. Viraemia suppression was greater than expected by additive interactions, thus suggesting synergic antiviral effects.[245]

Relapse rates after combined IFN-α and lamivudine treatment are also high. In one study, patient relapse occurred within 1–3 months of follow-up, reaching 75%.[246] Combination therapy of IFN-α and lamivudine is therefore only marginally better than either drug alone. It appears therefore that such treatment has no added benefit, in contrast to the findings in the woodchuck animal model.[245]

Combination therapy of IFN-α and famciclovir

This has also been attempted in a pilot study.[247] Of five patients treated for an overlapping period of 20 weeks with combination therapy, one lost HBV-DNA during treatment and the other during the follow-up period.

Combination therapies with two or more nucleoside analogues

This has also been tried. For example, 12 weeks of lamivudine plus famciclovir were shown to be more effective in reducing HBV-DNA than lamivudine alone.[248] During a 16 week follow-up period, relapses in the lamivudine monotherapy group were 67%, but none was observed in the combination group. Synergic effects between the two drugs have in addition been reported in the animal models.[249,250] A combination of penciclovir, lamivudine and adefovir in primary duck hepatocyte cultures had similar effects.[251] In contrast, lamivudine and famciclovir in combination with IFN-α were no

better than the combination of the two nucleoside analogues alone.[252]

Future treatments

Immunotherapy

Therapeutic vaccination. This is another approach that has been employed in an attempt to break tolerance and stimulate T-cell immune responses in chronic HBV carriers, using the licensed or newly developed vaccines, different adjuvants and by altering the route of administration.[4] Immunization with recombinant HBsAg particles from transgenic mice expressing either HBsAg alone or replicating the virus resulted in marked reduction in serum HBsAg levels, loss of HBeAg or even development of anti-HBs.[253,254] Pilot studies in chronic HBV patients suggested that standard HBV vaccination could lead to clearance or reduction of HBV replication in ~50% of such patients.[255,256] In a larger controlled study of 118 patients receiving either GenHevac B (Pre-S2/S), Recombivax (S) or no vaccine, HBeAg to anti-HBe seroconversion was seen in 13.3% of vaccinees versus 3.6% of controls after 6 months of follow-up. After 12 months of follow-up, the seroconversion rate gap between the two groups narrowed to 18.9% versus 12.5%. None of the patients lost HBsAg.[257]

Alum-based vaccines, as the current HBV vaccine, promote production of antibodies and a Th2 biased immune response. For effective therapeutic vaccination, however, both humoral and cytotoxic T-cell responses may be necessary to eradicate infected cells. The use of alternative adjuvants such as MF59 already tested in healthy adults may improve vaccine efficacy.[258] Preliminary results that appeared in abstract form suggest that 11 of 13 patients with chronic HBV developed an anti-HBs response to such a vaccine.[259] Another adjuvant of potential benefit is CpG DNA, a synthetic oligonucleotide that preferentially stimulates Th1 responses, with production of IL-12 and IFN-γ.[260] Immunization of transgenic animals with an HBsAg vaccine supplemented with CpG DNA led to clearance of serum HBsAg and development of anti-HBs, with concurrent down-regulation of HBV mRNA production in the liver. Adoptive transfer experiments of T-cells from such animals showed that they were able to partially control transgene expression in the liver and to clear the HBsAg from the sera of recipient transgenic mice, without an antibody requirement.[261] A CpG-containing HBsAg vaccine was shown to overcome hyporesponsiveness normally seen in immunized orang-utans.[262] It remains to be seen whether similar responses are observed in human trials.

Peptide-based T-cell vaccines have also been tested in patients chronically infected with HBV. A lipopeptide (CY-1899) containing a T-helper epitope from tetanus toxoid and a CTL epitope from HBV core (amino acids 18–27) was tested in 90 chronic HBV carriers by Heathcote *et al.*[263] The vaccine induced CTL activity, which was not sufficient to clear the infection. Similar experiments in woodchucks co-immunized with WHVsAg together with a peptide from sperm whale myoglobin led to production of anti-WHVs in all immunized animals. However, two of the animals with the highest antibody levels developed severe liver damage, and one of them died.[264] Care must therefore be exercised in the choice of T-helper epitopes.

DNA-based vaccines. Intramuscular injection of plasmids encoding HBV antigens is another novel approach to vaccination, which enables the expression of encoded proteins *in vivo*, in their native conformation and with the appropriate post-translational modifications. Moreover, such proteins are processed intracellularly and the correct epitopes are thus presented to the immune system. Plasmid DNA immunization results in the generation of humoral immune responses, but potent CD8+ CTL responses are also induced, as shown initially in mice using HBsAg- or HBcAg-expressing constructs.[265,266] Similar experiments in HBsAg transgenic mice induced persistent clearance of circulating HBsAg.[267,268] Moreover, adoptive transfer of HBsAg-primed spleen cells from DNA-immunized mice achieved control of transgene expression, in the absence of anti-HBs production.[269] Augmented immune responses have been obtained in mice by including in DNA constructs sequences encoding T-helper epitopes such as PADRE (pan-DR epitope),[270] and cytokines such as IL-2.[271]

DNA immunization has also been employed for prophylaxis in experiments carried out in animal models of hepadnaviral infection with encouraging results.[272,273] In addition, DNA vaccines have been evaluated for safety and induction of immune responses in naive primates such as chimpanzees[272,274] and rhesus macaques,[273] and shown to produce high anti-HBs titres. Similar experiments in Aotus monkeys were not so promising.[275] Nevertheless, a DNA vaccine in newborn chimpanzees was shown to protect from subsequent challenge in spite of poor anti-HBs responses.[276] Immunization with HBsAg-encoding plasmid DNA, followed by recombinant HBsAg-expressing canarypox as booster in a chimpanzee HBV carrier, resulted in a 400-fold reduction in serum HBV-DNA levels, over a long period of time. In contrast, HBsAg levels in serum remained constant.[277] In another study, an HBcAg-expressing retroviral vector was used to immunize three HBV carrier chimpanzees. One of the animals seroconverted from HBeAg to anti-HBe following an ALT flare, whereas the other two animals remained positive for HBeAg and viral load was unaffected, even though one of the animals had detectable HBcAg-specific CTL responses.[278]

A DNA vaccine against HBV has also been evaluated in healthy human volunteers using the PowderJect system to

deliver gold particles coated with plasmid DNA directly to skin cells. The vaccine proved safe, was well tolerated and produced preferentially Th1 helper cell responses. Humoral anti-HBs responses were however weak.[279] There are no published reports as yet on the use of such vaccines in chronic HBV carriers.

Molecular approaches

Antisense oligonucleotides

Antisense oligodeoxynucleotides (ODN) are synthetic DNA molecules that can inhibit gene expression within cells by binding to complementary mRNA sequences, thus preventing translation.[280] Phosphorothioate ODNs are nuclease resistant, so that they are still biologically active when they reach their intended site of action.[281] Early experiments in cells transiently or stably transfected with plasmids encoding HBV proteins or the whole genome indicated that ODNs were effective in inhibiting viral protein expression and viral replication.[282–286] Similar experiments in DHBV-infected ducks or avian cells in culture yielded promising results.[287–290] However, *in vivo* efficacy particularly in man will be dependent on efficient delivery of the ODNs to the liver and at sufficient concentration. Such an approach will be quite expensive in view of the possible lengthy period of treatment that will be required, as the cccDNA pool of infected hepatocytes will persist. This approach is therefore unlikely to be used as monotherapy.

Ribozymes

Ribozymes (ribonucleic acid enzymes) are naturally occurring RNA molecules that catalyse RNA sequence-specific cleavage and splicing.[291] The smallest of them, known as 'hammerhead' from their characteristic secondary structure shape, recognize a minimal target sequence for cleavage. RNA cleavage specificity is mediated by the ribozyme sequence, which is complementary to that of the target RNA, and flanking the catalytic sequence. A number of studies so far have demonstrated efficient cleavage of HBV mRNAs in *in vitro* experiments using transfected cells or cell-free systems.[292–296] Ribozyme activity has been demonstrated by targeting regions that included the encapsidation signal in pgRNA,[293] HBx RNA[295–298] and the poly(A) signal region of HBV.[214] Ribozymes are presently in Phase I/II clinical studies.

Other molecular approaches include the use of dominant-negative HBV core mutant proteins as inhibitors of nucleocapsid formation within hepatocytes,[299] and peptide aptamers also targeting the core protein.[300] These have been tested in the duck animal model[299] and in transfected cells *in vitro*,[299,300] and have been shown capable of inhibiting capsid formation and consequently HBV replication.

Conclusion

IFN-α or nucleoside analogue monotherapy treatments are effective in suppressing HBV replication, leading to HBeAg seroconversion, normalization of ALT levels, improvement in histology and in some cases even loss of HBsAg. However, such favourable outcomes are attainable in only about a third of those treated, at the best of times. In spite of this, certain groups of patients have benefited tremendously from the use of nucleoside analogues, such as those with decompensated cirrhosis and chronic HBV patients undergoing liver transplantation, when there was little hope for them before.

Patients who do not respond to monotherapy treatment protocols may benefit from combination therapies, as has been the case in HIV treatment. Drugs acting through different antiviral mechanisms may supplement each other, by additive or synergic effects. Such future therapies may include immunotherapies and molecular approaches, as discussed above. If successful, these may reduce the duration and cost of treatment, lessen the impact of side-effects, and more importantly prevent the emergence of drug-resistant variants of the virus. Combination therapies in Phase II and III clinical studies at the moment will hopefully prove successful and lead to the selection of the optimum cocktail of drugs and duration of treatment.

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Hepatocellular carcinoma

A concise guide to its status and management

Scott C. Ulmer, MD

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CME learning objectives

- To perceive the growing importance of hepatocellular carcinoma in the United States
- To learn the diagnostic tests available for hepatocellular carcinoma and their indications
- To understand limitations of treatment and the need for improved screening and prevention in hepatocellular carcinoma

This is the third of three articles on gastrointestinal cancers

This page is best viewed with a browser that supports tables

Preview: Hepatocellular cancer is one of the most common cancers in the world. It is also one of the most deadly, with a 5-year survival rate of less than 5% without treatment. And investigators have added a note of alarm to this already grim scene--the incidence of the disease is on the rise in the United States. Primary care physicians have a critical role because they are often the first to see these patients. In this article, Dr Ulmer discusses how increased knowledge of the pathogenesis of hepatocellular carcinoma and advances in detection, prevention, and treatment have made possible discovery of this aggressive tumor at potentially treatable stages.
Ulmer SC. Hepatocellular carcinoma: a concise guide to its status and management. Postgrad Med 107(5):117-24

Any chronic inflammatory liver disease has the potential to induce hepatocellular carcinoma, but the pathophysiologic process most commonly associated with the disease is cirrhosis, found in up to 80% of cases (1). However, knowledge of all possible sources is important, considering that 20% of cases are due to noncirrhotic, nonviral causes. Whether cirrhosis itself or the mechanism underlying cirrhosis is responsible for malignant transformation of hepatocytes is not known.

Certain viral, environmental, and hereditary causes of cirrhosis have a strong correlation with hepatocellular carcinoma. Chronic viral hepatitis as a cause of cirrhosis and hepatocellular carcinoma is well known. Hepatitis B virus infection is the leading cause of chronic liver disease and hepatocellular carcinoma around the world. About 350 million people worldwide have chronic hepatitis B, with the highest prevalence found in regions that have the highest rates of hepatocellular carcinoma (2). In the United States, hepatitis B virus infects about 1.2 million people and hepatitis C virus about 4 million people (3). Hepatitis C virus RNA is found in about 65% of patients who test negative for hepatitis B surface antigen at diagnosis of their hepatocellular carcinoma (4).

Alcohol use is also a common cause of cirrhosis, which can indirectly lead to hepatocellular carcinoma. However, a direct carcinogenic effect of alcohol on the liver has not been proved.

Certain substances derived from plants, industrial pollutants, and synthetic pharmaceutical agents have been found to cause hepatocellular carcinoma in animals and, as proved through epidemiologic studies, in humans. For example, when present in detectable levels, aflatoxin B (a mycotoxin in inappropriately stored grain) confers an odds ratio for hepatocellular carcinoma of 5.5 (5). Vinyl chloride is the most studied of the industrial carcinogens. Estrogens and androgens, as found in oral contraceptives and anabolic steroids, have been found to be carcinogenic in rodents.

Hemochromatosis carries a relative risk of more than 200 for hepatocellular carcinoma, which can occur without cirrhosis (6). Alpha$_1$-antitrypsin deficiency and primary biliary cirrhosis are also associated with the disease.

A disturbing trend

The incidence of hepatocellular carcinoma varies greatly with geographic location, ethnic background, and sex. For instance, incidence rates among men in sub-Saharan Africa and Asia may be 20 times higher than those among men in the United States (7). This disparity between countries is probably related to endemic rates of viral hepatitis and environmental carcinogens (eg, aflatoxins). The male-female ratio is about 4:1 (1).

A concerning trend has recently been noted in developed

countries. For unknown reasons, the incidence of the disease is rising (8,9). In the United States, the incidence of histologically proved hepatocellular carcinoma increased from 1.4/100,000 population in the period from 1976 to 1980 to 2.4/100,000 population in the period from 1991 to 1995 (9). Figure 1 (not shown) shows these trends over time in men versus women and blacks versus whites. Although the rise may be due to detection bias, reasons for it are unclear. One proposed explanation is widespread transmission of viral hepatitis during the late 1960s through injecting drug use and unscreened blood product transfusions (9).

The United Kingdom and France have also documented increases in hepatocellular carcinoma mortality rates from 1979 to 1994 (10,11). One encouraging study (12) has shown that through aggressive hepatitis B vaccination, hepatocellular carcinoma incidence rates in Taiwanese children declined significantly during the first 10 years of the program.

Patient presentation

Hepatocellular carcinoma should be considered in any patient with chronic liver disease whose clinical status indicates sudden decompensation. Unfortunately, the disease is often clinically silent until it is well advanced or tumor diameter exceeds 10 cm (13). The most common presenting symptoms and physical examination findings are summarized in table 1 (1). A few patients present with paraneoplastic syndrome, and the most common and most significant manifestations are erythrocytosis, hypercalcemia, hypoglycemia, carcinoid syndrome, dysfibrinogenemia, cryoglobulinemia, and hypercholesterolemia (14).

Table 1. Common presenting symptoms and physical examination findings in hepatocellular carcinoma

Finding	Average incidence (%)
Symptom	
Abdominal pain	91
Abdominal swelling	43
Weight loss	35
Weakness	31
Feeling of fullness and anorexia	27
Vomiting	8
Jaundice	7
Physical examination	
Hepatomegaly	89
Splenomegaly	65
Ascites	52
Jaundice	41
Fever	38
Hepatic bruit	28

Data from Flickinger et al (1).

Diagnostic approach

Anyone with chronic liver disease whose status is declining should be assessed thoroughly in a broad differential diagnosis approach. Basic laboratory evaluation should include a complete blood cell count, blood chemistries, transaminase levels, albumin level, and prothrombin time. To help detect hepatocellular carcinoma, alpha-fetoprotein (AFP) should be measured; a level exceeding 500 micrograms/L is considered a positive finding (14). Unfortunately, in the US population, the sensitivity of an elevated AFP level is only 60% (1). More sensitive tests, such as soluble interleukin-2 receptor levels, are currently being developed and results are encouraging (15).

Paracentesis should be performed if imaging studies or physical examination reveals ascites. Bloody ascites is found in 20% of patients with hepatocellular carcinoma, and during paracentesis, fluid can be obtained for cytologic evaluation (14).

Imaging studies are an important part of diagnosing hepatocellular carcinoma, but which imaging procedure is used differs in the United States versus the rest of the world. Most studies screening for hepatocellular carcinoma have been performed outside the United States where ultrasound combined with AFP measurement achieves greater sensitivity and specificity than other methods. In contrast, in a recent study of US patients with advanced cirrhosis, spiral computed tomography (CT) was found to have an 88% sensitivity for detecting hepatocellular carcinoma, which was significantly better than the 59% sensitivity of abdominal ultrasound (16). Hypotheses to explain the reduced sensitivity of ultrasound are that in US patients the liver is more steatotic because of alcohol consumption and harder to visualize because of body habitus and that less-skilled technicians perform the procedure compared with physician ultrasonographers in Asia.

Whatever the explanation, emerging studies support multiple-phase abdominal CT as the imaging procedure of choice, despite current high costs and limited availability. On the basis of these findings, the University of Utah Hospital and Clinics evaluates patients who have cirrhosis or suspected hepatocellular carcinoma with CT consisting of three phases: (1) scanning before the intravenous bolus of contrast is given, (2) scanning shortly after contrast administration, and (3) routine portal venous phase scanning of the entire abdomen, including the liver. Hepatocellular carcinoma is seen most commonly in the second--the hepatic arterial--phase, because the lesions are often hypervascular (figure 2: not shown).

Magnetic resonance imaging is less sensitive than angiographically assisted helical CT in diagnosing hepatocellular carcinoma and is currently used to further characterize the disease within a nodular liver (17). Fine-needle biopsy of the mass should always be considered when the diagnosis is unclear, but theoretically, it carries risks for percutaneous needle tract seeding of tumor and bleeding.

Staging and prognosis

Accurate staging is critical for determining the prognosis and therapeutic approach in hepatocellular carcinoma. The International Union Against Cancer (Union Internationale Contre le Cancer, UICC) has developed a TNM classification for the disease. A retrospective study evaluated survival in North American patients with all stages of hepatocellular carcinoma and found median survival time to be 10 months (18). However, 35% of patients with limited-stage disease who were treated aggressively (figure 3: not shown) attained survival of 5 years (18).

Treatment methods

As mentioned, treatment of hepatocellular carcinoma can drastically alter survival in appropriately selected patients. In those with small tumors (<2 cm in diameter), limited stage I or II disease, and good hepatic function, surgical resection is the treatment of choice. It can achieve 5-year survival rates as high as 60% to 70% and, rarely, cure the disease (5).

Mazzaferro and associates from the National Cancer Institute, Milan, Italy (19), found that similar or even better results could be obtained with liver transplantation. This study found 4-year survival rates of 75% and recurrence-free survival rates of 83% with transplantation in patients with small but unresectable tumors. Although results of this Italian trial are encouraging, similar trials in US patients have not found survival rates after liver transplantation to match those after surgical resection (20). Therefore, in the United States, liver transplantation for hepatocellular carcinoma is indicated only in patients who have unresectable tumors less than 5 cm in diameter, focal tumor recurrence after resection, or hepatic failure.

The list of treatment methods for hepatocellular carcinoma is vast and growing. Specialized procedures, including transcatheter arterial embolization, chemoembolization, lipoidal-targeted chemotherapy, and transcatheter oily chemoembolization, can improve survival rates in patients with unresectable cancer. However, these techniques are used mainly in Asia as adjunctive methods in patients who are eligible for resection or transplantation. For patients who are poor surgical candidates but have small numbers of tumors, all less than 3 cm in diameter, percutaneous ethanol injection achieves a 3-year survival rate that is similar to that of resection, plus it is relatively inexpensive and widely available.

Because of their excessive toxicity and lack of survival benefit, systemic chemotherapy and radiation therapy are not considered efficacious approaches to hepatocellular carcinoma.

Prevention and screening

Given the poor prognosis and lack of effective therapies for hepatocellular carcinoma, programs for prevention are desperately needed. Nonspecific measures to ensure sterile needles, safe laboratory practices, a clean donor blood supply, good general hygiene, and effective public health policies should be every country's priority. As mentioned, Taiwan has instituted universal hepatitis B vaccination for children and has seen a decline in the incidence of hepatocellular carcinoma from 0.7/100,000 children between 1981 and 1986 to 0.36/100,000 children between 1990 and 1994 (12). Other potential interventions include use of lamivudine, interferon, and other antiviral agents for treatment of chronic active viral hepatitis. However, large randomized trials have yet to prove that these methods reduce hepatocellular carcinoma (21,22).

Screening for hepatocellular carcinoma has limitations. Cirrhosis and hepatocellular carcinoma arising out of cirrhosis are often clinically silent. Available screening tests are expensive and not 100% sensitive, and often, survival is not affected once hepatocellular carcinoma is discovered.

Nonetheless, screening has become the standard of care and may be cost-effective when patients are carefully selected. High-risk features for development of hepatocellular carcinoma are cirrhosis and chronic active hepatitis B or chronic hepatitis C (23). Patients with either of these variables should be screened every 6 months with AFP measurement and abdominal helical CT (or abdominal ultrasound if CT is unavailable).

In high-risk patients, 40% to 60% of hepatocellular carcinomas identified are small and in the treatable stage (24). No screening tests have been found to be effective in patients who do not meet the high-risk criteria for development of hepatocellular carcinoma.

Summary

Although common worldwide, hepatocellular carcinoma is relatively rare in the United States. However, for unknown reasons, the incidence is rising. Multiple causes exist, but chronic viral hepatitis in the setting of cirrhosis is probably the most common. Despite limitations, AFP measurement and multiple-phase abdominal CT are the most sensitive tests for diagnosis. Surgical resection and liver transplantation are at present the only treatment options that offer potential for long-term survival or cure in limited-stage hepatocellular carcinoma. Otherwise, the prognosis is poor, and 1-year survival is rare. Future efforts should focus on improving detection of early-stage disease and improving preventive measures to reduce viral hepatitis infection,

transmission, and progression.

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For a helpful guide to electronic and print resources on gastrointestinal cancers for physicians and patients, see the Resource Guide in this issue.

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Alpha Interferon Inhibits Hepatitis C Virus Replication in Primary Human Hepatocytes Infected In Vitro

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Chronic hepatitis C is a common cause of liver disease, the complications of which include cirrhosis and hepatocellular carcinoma. Treatment of chronic hepatitis C is based on the use of alpha interferon (IFN-α). Recently, indirect evidence based on mathematical modeling of hepatitis C virus (HCV) dynamics during human IFN-α therapy suggested that the major initial effect of IFN-α is to block HCV virion production or release. Here, we used primary cultures of healthy, uninfected human hepatocytes to show that: (i) healthy human hepatocytes can be infected in vitro and support HCV genome replication, (ii) hepatocyte treatment with IFN-α results in expression of IFN-α-induced genes, and (iii) IFN-α inhibits HCV replication in infected human hepatocytes. These results show that IFN-α acts primarily through its nonspecific antiviral effects and suggest that primary cultures of human hepatocytes may provide a good model to study intrinsic HCV resistance to IFN-α.

Hepatitis C has emerged in recent years as a common cause of liver disease, and an estimated 170 million people are thought to be infected worldwide. Hepatitis C virus (HCV) infection is characterized by viral persistence and chronic liver disease in approximately 80% of cases. The complications of chronic hepatitis C include cirrhosis in 20% of cases and hepatocellular carcinoma, which has an incidence of up to 4 to 5% per year in patients with cirrhosis. Hepatitis C-related end-stage liver disease is now the principal indication for liver transplantation in industrialized countries (2).

HCV is a single-strand positive-sense RNA virus belonging to the family *Flaviviridae*. Translation of its only open reading frame leads to the synthesis of a single polyprotein which is secondarily cleaved by both host and viral proteases, giving rise to structural and nonstructural proteins (22). The nonstructural protein 5B (NS5B) is an RNA-dependent RNA polymerase (RdRp). The mechanisms of HCV replication in host cells are poorly understood. It is thought that RdRp, along with other nonstructural proteins, the HCV RNA template, and host cell factors, forms a replication ribonucleoprotein complex associated with perinuclear membranous structures that would be the site of RNA replication (13, 43). By analogy with other members of the family *Flaviviridae*, the replication strategy within this complex would be the production of a negative-strand copy of the RNA genome, which would in turn serve as a template for the production of progeny positive-strand RNA. Indeed, negative-strand HCV RNA has been detected in various cells and tissues supporting HCV replication (1, 18, 20, 28, 39, 40).

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HCV RdRp, like other viral RNA polymerases, has a high error rate, with misincorporation frequencies averaging about 10^{-4} to 10^{-5} per base site, in the absence of a proofreading mechanism. As a result, mutations accumulate in newly generated HCV genomes. Most mutant viral particles are replication deficient, but some propagate efficiently. The fittest infectious particles are selected continuously on the basis of their replication capacities and environmental selective pressures (mainly the host immune response). This explains why each infected individual harbors a pool of genetically distinct but closely related HCV variants referred to collectively as a quasispecies (24, 45).

Treatment of chronic hepatitis C is aimed at preventing complications, especially cirrhosis and hepatocellular carcinoma. It is currently based on subcutaneous injection of recombinant alpha interferon (IFN-α) three times a week or of its pegylated form (i.e., IFN-α combined with polyethylene glycol) once a week. The antiviral efficacy of IFN-α is potentiated by ribavirin, a nucleoside analog with an unknown mechanism of action (2, 10, 21, 25, 26, 36, 47). Combination therapy with pegylated IFN-α plus ribavirin for 24 to 48 weeks leads to permanent viral clearance in 42 to 82% of patients according to HCV genotype; other patients have ongoing viral replication and remain at risk of disease progression [23; M. W. Fried, M. L. Shiffman, R. K. Reddy, C. Smith, G. Marinos, F. L. Goncales, Jr., et al., Gastroenterology **120**(Suppl. A):55, 2001].

After subcutaneous administration, IFN-α specifically binds to high-affinity receptors at the surface of target cells. IFN-α binding to its receptor triggers a cascade of intracellular reactions, leading to activation of numerous IFN-induced genes (11, 31, 38, 41, 44). The products of these genes mediate the cellular actions of IFN-α. As IFN-α binds to surface receptors of immune cells, it has immunomodulatory effects (34, 42). IFN-α binding to various cells also induces numerous proteins

and enzymatic pathways involved in establishing a non-virus-specific antiviral state through distinct but complementary mechanisms (3, 12, 27, 37, 49). Specific IFN-α binding to human hepatocytes and subsequent activation of IFN-α-induced genes leading to the establishment of an antiviral state have not yet been documented.

Recently, indirect evidence based on mathematical modeling of HCV dynamics during human IFN-α therapy suggested that the major initial effect of IFN-α is to block HCV virion production or release (30). Inhibition of HCV replication by both IFN-α and IFN-β has been observed in a human lymphocytic cell line supporting HCV genome replication (40). More recently, IFN-α was shown to inhibit subgenomic HCV RNA replication in HuH-7 human hepatoma cell lines (8, 9) and full-length HCV RNA replication in a binary expression system in CV-1 monkey kidney cell lines (4). Nevertheless, IFN-α blockade of HCV replication has never been demonstrated in healthy, uninfected human hepatocytes.

MATERIALS AND METHODS

Primary cultures of human hepatocytes. Healthy human hepatocytes were isolated from surgical liver resection specimens from 15 liver donors (Table 1). The two-step collagenase perfusion method was used (35). Cell viability, assessed by the trypan blue exclusion test, was greater than 85%. Hepatocytes were plated at confluence (14×10^4 cells/cm^2) in 60- or 35-mm-diameter culture dishes precoated with type I collagen (Iwaki Glass, Chiba, Japan) in a total volume of 3.0 or 1.5 ml of standard culture medium consisting of a mixture of Williams' E medium and Ham F12 medium (1:1 volume) supplemented as recommended elsewhere (14) and containing 5% fetal calf serum to favor cell attachment. After 4 h, the standard medium was replaced with 3.0 or 1.5 ml of a previously described modified serum-free long-term culture medium (6, 17). This medium was renewed after 24 h and then every 48 h. The cultures were maintained in humidified 95% air–5% carbon dioxide at 37°C.

In vitro infection of primary cultures of human hepatocytes. Serum samples from nine patients with chronic HCV genotype 1 infection were used for cell infection (the HCV subtypes and viral loads are shown in Table 1). Three days after plating (to permit cell recovery from isolation), the cells were infected in vitro by overnight incubation with 25 μl of HCV-positive serum in 3.0 ml of medium. The cells were then washed three times with 3.0 ml of fresh medium, and the cultures were continued under normal conditions in long-term culture medium. Cells and culture medium were collected at various times during culture and stored at −80°C. All experiments were carried out in duplicate.

Treatment of primary hepatocyte cultures with IFN-α. Recombinant IFN-α 2a (Roferon-A; Hoffmann-La Roche, Basel, Switzerland) was used at final concentrations of 500 to 10,000 U/ml. IFN-α treatment was started at the time of HCV infection in most experiments and 3 days after infection in some cases. IFN-α was replaced on a daily basis as the culture medium was changed. All experiments were carried out in duplicate.

Western blot analysis of IRF-1 and PKR induction. Total protein corresponding to 400,000 cells was extracted in Laemmli buffer, electrophoresed on 12.5% polyacrylamide gels, and then transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.). Western blotting was performed using the Santa Cruz polyclonal antibodies against interferon-responsive factor 1 (IRF-1) (kindly provided by N. Mechti, INSERM U475, Montpellier, France) and a monoclonal antibody against human RNA-dependent protein kinase (PKR) (kindly provided by E. Meurs, Institut Pasteur, Paris, France). The proteins were visualized by using an enhanced chemiluminescence detection method (Amersham Pharmacia, Abingdon, United Kingdom), and the blots were analyzed with a National Institutes of Health image analyzer in order to measure the amounts of induced proteins.

Cellular RNA extraction and strand-specific *rTth* RT-PCR. At the time of cell harvest, the medium was removed, and the cultures were washed three times with cold phosphate-buffered saline. RNA was purified from 4×10^6 or 2×10^6 cultured hepatocytes or from 100-μl samples of HCV-positive sera, using a guanidium isothiocyanate-acid phenol extraction procedure (RNABLE; Eurobio, Les Ulis, France). Precipitated RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated water and quantified by UV spectrum analysis. Extracted RNA was analyzed with a modification of the previously described strand-specific

TABLE 1. Characteristics of the primary cultures of human hepatocytes and the corresponding HCV-positive sera used for inoculation and summary of experiments and results

Culture	Hepatocyte characteristics			Infecting serum			IFN-α treatment	IRF-1 and PKR induction by IFN-α	IFN-α toxicity	(+) and (−) HCV RNA strands[f]	Accumulation of (+) HCV RNA[g]	Effect of IFN-α on genetic evolution
	Donor sex[a]	Donor age (yr)	Origin	Serum	Genotype	Viral load[d]						
FT141	F	52	Left lobe	S23	1b	7.3	Yes	NT[e]	NT	Present	NT	Reduced
FT143	F	64	Right lobe	S34	1b	5.6	Yes	NT	NT	Present	NT	Unchanged
FT144	M	60	Right lobe	S27	1b	>7.4	Yes	NT	NT	Present	NT	NT
FT147	M	54	Right lobe	S26	1b	6.7	Yes	NT	NT	Present	NT	Unchanged
FT154	M	61	Segments VI-VII	S23	1b	7.3	Yes	NT	No toxicity	Present	NT	NT
FT155	F	55	Left lobe	S20	1b	>7.4	Yes	NT	No toxicity	Present	NT	Reduced
FT156	M	77	Right lobe	S17	1b	6.9	Yes	NT	No toxicity	Present	NT	Reduced
FT161	M	Unknown	Deceased organ donor	S42	1b	5.2	Yes	NT	NT	Present	NT	NT
FT164	F	47	Right lobe	NI[b]	NA[c]	NA	Yes	Present	NT	NT	NT	NT
FT171	M	48	Segment V-VII	NI	NA	NA	Yes	Present	NT	NT	NT	NT
FT172	F	57	Left lobe	S42	1b	5.2	Yes	Present	NT	Present	Present	NT
FT168	F	75	Left lobe	S34	1b	5.6	No	NT	NT	Present	Present	NT
FT187	M	66	Segment VI-VII	S155	1b	6.0	Yes	NT	NT	Present	Present	NT
FT189	M	48	Left lobe	S155	1a	6.0	No	NT	NT	Present	Present	NT
FT195	M	17	Deceased organ donor	S155	1a	6.0	No	NT	NT	Present	Present	NT

[a] M, male; F, female.
[b] NI, no infectious challenge.
[c] NA, not applicable.
[d] Viral load in $\log_{10}$ HCV RNA international units/milliliter
[e] NT, not tested.
[f] Positive-sense (+) and negative-sense (−) HCV RNA strands by the qualitative, nonquantitative, strand-specific *rTth* RT-PCR-based assay.
[g] Accumulation of positive-sense (+) HCV RNA by the quantitative real-time PCR-based assay.

rTth reverse transcription-PCR (RT-PCR) assay (7). Primers located in the HCV 5′ noncoding region, including antisense primer HCV-I (5′-TGG[A]TGCACG-GTCTACGAGACCTC-3′, nucleotides [nt] 342 to 320) and sense primer HCV-II (5′-CACTCCCCTGTGAGGAACT-3′, nt 38 to 56) (19), were used. In the positive-strand HCV RNA assay, 1 μg of cellular RNA (corresponding to approximately 6×10^4 cells) in 10 μl of diethyl pyrocarbonate-treated water was layered with mineral oil and heated at 95°C for 1 min. The temperature was then lowered to 70°C, and a 20-μl reaction mixture containing 50 ng of primer HCV-I, 1× RT buffer (Applied Biosystems, Foster City, Calif.), 1 mM $MnCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 5 U of *rTth* enzyme (Applied Biosystems) was prepared for cDNA synthesis. Primer annealing was performed at 60°C for 2 min, followed by the RT reaction at 70°C for 20 min. In order to inactivate the RT activity of *rTth*, Mn^{2+} was chelated with 40 μl of a mixture containing 8 μl of 10× EGTA chelating buffer (Applied Biosystems). Forty microliters of the prewarmed (70°C) PCR mixture containing 50 ng of primer HCV-II and 3.75 mM $MgCl_2$ was added. PCR was performed on the GeneAmp PCR-System 9600 apparatus (Applied Biosystems) and consisted of the following: (i) an initial denaturation step of 1 min at 94°C; (ii) 50 cycles, with 1 cycle consisting of 15 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and (iii) a final extension step of 7 min at 72°C. PCR products were analyzed by agarose gel electrophoresis. The negative-strand HCV RNA assay was performed by the same procedure, except that the primers were used in reverse order.

Real-time PCR quantification of positive- and negative-strand HCV RNA. Both positive- and negative-strand HCV RNAs were quantified by means of a real-time PCR assay using the LightCycler instrument and technology (Roche Applied Science, Indianapolis, Ind.) and SYBR green I dye for detection. The primer pair was located in the HCV 5′ noncoding region and included antisense primer KY78 (5′-CTCGCAAGCACCCTATCAGGCAGT-3′, nt 311 to 288) and sense primer KY80 (5′-GCAGAAAGCGTCTAGCCATGGCGT-3′, nt 68 to 91) (46). One microgram of cellular RNA was used for cDNA synthesis in a 20-μl reaction mixture containing 5 U of *rTth* polymerase and 1 μM RT primer. Primer HCV-I was used for positive-strand cDNA synthesis, and primer HCV-II was used for negative-strand cDNA synthesis. In addition, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as an internal control for the quality of extracted cellular RNA, with RT using primer GAPDH-31 (5′-GCCTGCTTCACCACCTTCTTG-3′, nt 869 to 849). cDNA was synthesized at 70°C for 20 min in all instances, and generated cDNA was purified with the HighPure PCR product purification kit (Roche Applied Science) in a 50-μl volume. Positive- and negative-strand HCV PCR amplifications were performed with 3 μl of purified cDNA in a 10-μl reaction mixture containing 1 μl of LightCycler-FastStart DNA Master SYBR green (Roche Applied Science) and 0.5 μM (each) HCV primer KY78 and KY80. PCR consisted of an initial denaturation step of 6 min at 95°C, followed by 45 cycles, with 1 cycle consisting of 15 s at 95°C, 5 s at 70°C, and 15 s at 72°C. All the samples were analyzed in triplicate. PCR amplification of GAPDH mRNA used primers GAPDH-51 (5′-ACAGTCCATGCCATCACTGCC-3′, nt 603 to 624) and GAPDH-31. One microliter of purified cDNA was used in 10 μl of mixture containing 1 μl of LightCycler-FastStart DNA Master SYBR green and 0.5 μM (each) primer. DNA was quantified in real time during the PCR by measuring fluorescent dye incorporation into PCR products at 530 nm. At the end of each run, a DNA melting step was performed, and the fusion curve was recorded to control for the homogeneity and quality of amplified DNA. In each run, 10-fold serial dilutions of synthetic positive- and negative-strand RNAs were tested in duplicate to establish a standard curve to calculate the amount of positive- and negative-strand HCV RNA in each sample. Tenfold serial dilutions of purified GAPDH mRNA amplicons were tested in duplicate to quantify GAPDH mRNA in each sample. The measured amounts of HCV RNA were normalized to the amount of GAPDH mRNA in each sample, and the results were expressed per culture plate (4×10^6 or 2×10^6 cells).

Generation of HCV quasispecies sequences. Extracted RNAs were reverse transcribed at 42°C for 60 min by using 70 ng of primer ASPR per μl (5′-AGC TCCGCCAAGGCAGAAGACAC-3′, nt 7347 to 7369) in the presence of 8 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). The first nested PCR round was performed using 5 pmol of degenerated sense primer HC378-1b (5′-TCCCRTGYGAGCCYGAACCG-3′, nt 6808 to 6828) and antisense primer MKed (5′-TTCCARGACTCTARCART-3′, nt 7193 to 7234) with *Pwo* high-fidelity DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). The second-round PCR used sense primer NS5A-S (5′-CCCACAT TACAGCAGAGACGGC-3′, nt 6865 to 6986) and antisense primer WARid (5′-GGRTTGTARTCCGGSCGYGCCCATA-3′, nt 7189 to 7213). After denaturation for 5 min at 94°C, the two PCR rounds comprised 30 cycles (1 cycle consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) and a final elongation step at 72°C for 5 min. Amplified products were analyzed by electrophoresis through 2% agarose gel and staining with ethidium bromide.

PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen BV, Gröningen, The Netherlands), according to the manufacturer's protocol. They were then ligated into the M13 vector, and recombinant plasmid DNA was transformed into competent *Escherichia coli* cells according to the manufacturer's protocol. Transformants were grown on plates containing brain heart infusion and ampicillin. Cloned DNA was reamplified by using both sense and antisense M13-specific primers in the presence of high-fidelity *Pwo* DNA polymerase. PCR products were purified with Microcon 100 before sequencing (Millipore, Dublin, Ireland).

After cloning and PCR amplification of 20 clones per time point, each clone was sequenced with the Cy5.0/Cy5.5 dye primer kit (Visible Genetics, Inc., Toronto, Canada) on a LongRead Tower automated DNA sequencer (Visible Genetics, Inc.) according to the manufacturer's instructions. The sequencing primers were the labeled upstream and downstream PCR primers.

Genetic and phylogenetic analyses of HCV quasispecies evolution. Nucleotide sequences were aligned using the CLUSTAL X program. Distances between pairs of sequences were calculated by using the DNADIST module in the PHYLIP package version 3.572 (distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle). Distance calculation was based on a Kimura two-parameter distance matrix with a transition-to-transversion ratio of 4.0. The means ± standard errors of the means (SEMs) of within-sample genetic distances were calculated for the inocula, and the means ± SEMs for between-sample genetic distances were calculated on the basis of distances between pairs of inocula (day 0) and postculture (day 8 of infection) sequences. Accumulation of synonymous and nonsynonymous substitutions per synonymous and nonsynonymous site, respectively, was calculated with the Jukes-Cantor correction for multiple substitutions, using the MEGA program (15). Statistical comparisons were made using a *t* test.

The PHYLIP program, version 3.572, was used to construct phylogenetic trees by means of the neighbor-joining method with a sequence matrix determined by the two-parameter method of Kimura. Patient viral sequence trees were constructed with nucleotide sequences. Phylogenetic analyses of all viral sequences generated in this study showed distinct clusters of viral sequences corresponding to each patient (data not shown), indicating the absence of PCR cross-contamination.

RESULTS

HCV replicates in primary cultures of healthy human hepatocytes infected in vitro. We prepared primary cultures of healthy human hepatocytes from 15 HCV-seronegative donors (Table 1). Ferrini et al. previously showed that healthy human hepatocytes retain a differentiated phenotype for at least 35 days under the conditions used here (6). Serum samples from nine patients chronically infected with HCV genotype 1 (subtype 1a or 1b) who had never been treated were used for in vitro infection of 13 of the 15 primary hepatocyte cultures (Table 1). Cultures were infected 3 days after plating and harvested at various times between 3 and 12 days of culture for extraction of total cellular RNA. Two complementary assays were used to study positive- and negative-sense HCV RNA strands in the inocula and infected hepatocyte cultures. The first assay is a highly sensitive qualitative (i.e., nonquantitative) detection assay, based on a modification of our previously described strand-specific *rTth* RT-PCR assay (7). As shown in Fig. 1a and b, the assay detects 0.1 fg of the correct RNA strand (i.e., 3×10^2 molecules), whereas at least 1 to 10 pg of the incorrect RNA strand is required to obtain a detectable signal. The second assay is a quantitative assay based on real-time PCR with the LightCycler technology allowing quantification of positive- and negative-sense HCV RNA strands in both the inocula and hepatocyte cultures (Fig. 1c). As shown in Fig. 1c, the tested interval from 3.5 to 5.5 log HCV RNA copies per capillary was within the dynamic range of quantification of

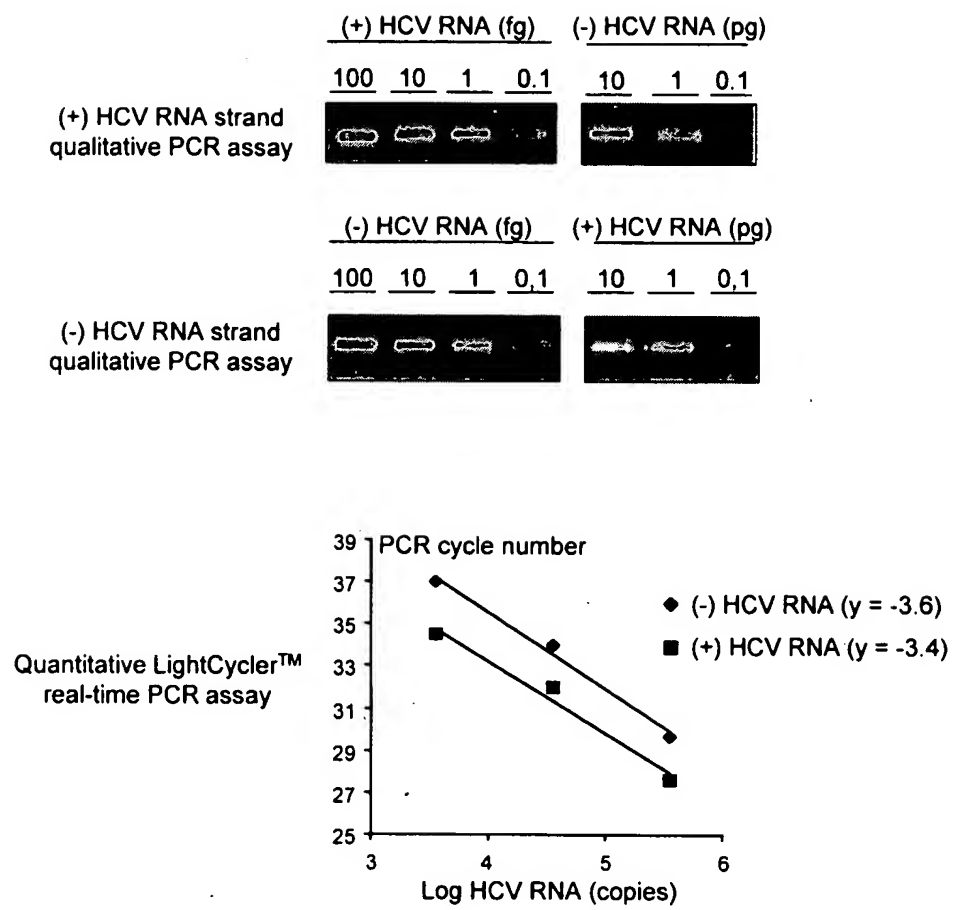

FIG. 1. Characteristics of the strand-specific HCV RNA assays used in this study. (a) Strand specificity of the positive-strand-specific HCV RNA *rTth* RT-PCR assay. Decreasing amounts of positive-strand (+) HCV RNA (100, 10, 1, and 0.1 fg) and of negative-strand (-) HCV RNA (10, 1, and 0.1 pg) synthesized from an appropriate plasmid were subjected to the *rTth* RT-PCR assay. The products were analyzed by agarose gel electrophoresis. (b) Strand specificity of the negative-strand-specific HCV RNA *rTth* RT-PCR assay. Decreasing amounts of negative-strand HCV RNA (100, 10, 1, and 0.1 fg) and positive-strand HCV RNA (10, 1, and 0.1 pg) synthesized from the same plasmid as for panel a were analyzed by the same procedure. (c) Range of linear quantification of the quantitative assay based on real-time PCR using the LightCycler technology and SYBR green I dye for detection. The range of linear quantification of the assay was studied by testing 10-fold serial dilutions of synthetic positive- and negative-sense HCV RNA strands after RT at 70°C with the *rTth* polymerase. Each point is the mean of three experimental values for each dilution. *y* is the slope of the linear plots.

the assay. The quantitative assay is less sensitive for HCV RNA strand detection than the qualitative assay, with a lower detection cutoff of 1 to 2 log HCV RNA copies per milliliter higher than the latter. The results are summarized in Table 1.

As expected, the positive-sense RNA strand was the only form of HCV RNA present in the inoculum. We thus used detection of the positive- and negative-sense RNA strands with the qualitative assay as a marker of HCV replication in hepatocyte cultures. Both positive- and negative-sense RNA strands were detected in the cultures. In culture FT147 infected with serum S26 (Fig. 2a), positive RNA strands were detected on day 1 postinfection and were still present in the last plate harvested. The negative-sense RNA strand was detected on day 2 postinfection and remained detectable up to day 10, proving viral replication in the culture. We observed the presence of positive-strand HCV RNA throughout the culture period and persistent expression of negative-strand HCV RNA in the other cultures infected with different sera (Table 1 and data not shown).

HCV replication in hepatocyte cultures was further supported by the accumulation of HCV RNA strands, as measured by real-time quantitative RT-PCR. Indeed, we observed a significant increase in the amount of both positive- and negative-sense HCV RNA strands in culture FT172 infected with serum S42 (Fig. 3a). A similar increase in the amount of positive-strand HCV RNA was observed in cultures FT189 and FT195, both infected with S155, but the total amount of positive-strand HCV RNA was smaller in these cultures at the various time points, suggesting less replication than that in culture FT172 (Fig. 3b and c). The negative-strand HCV RNA also accumulated in culture FT172, but the amount of negative-strand HCV RNA was consistently smaller than the amount of positive-strand HCV RNA on days 3 and 5 (Fig. 3a). This explains why, in cultures FT189 and FT195, negative-strand HCV RNA was not detected with the quantitative assay, whereas it was detected with the more sensitive qualitative assay; i.e., its amount was below the detection cutoff of the quantitative assay. Similar results were obtained with culture

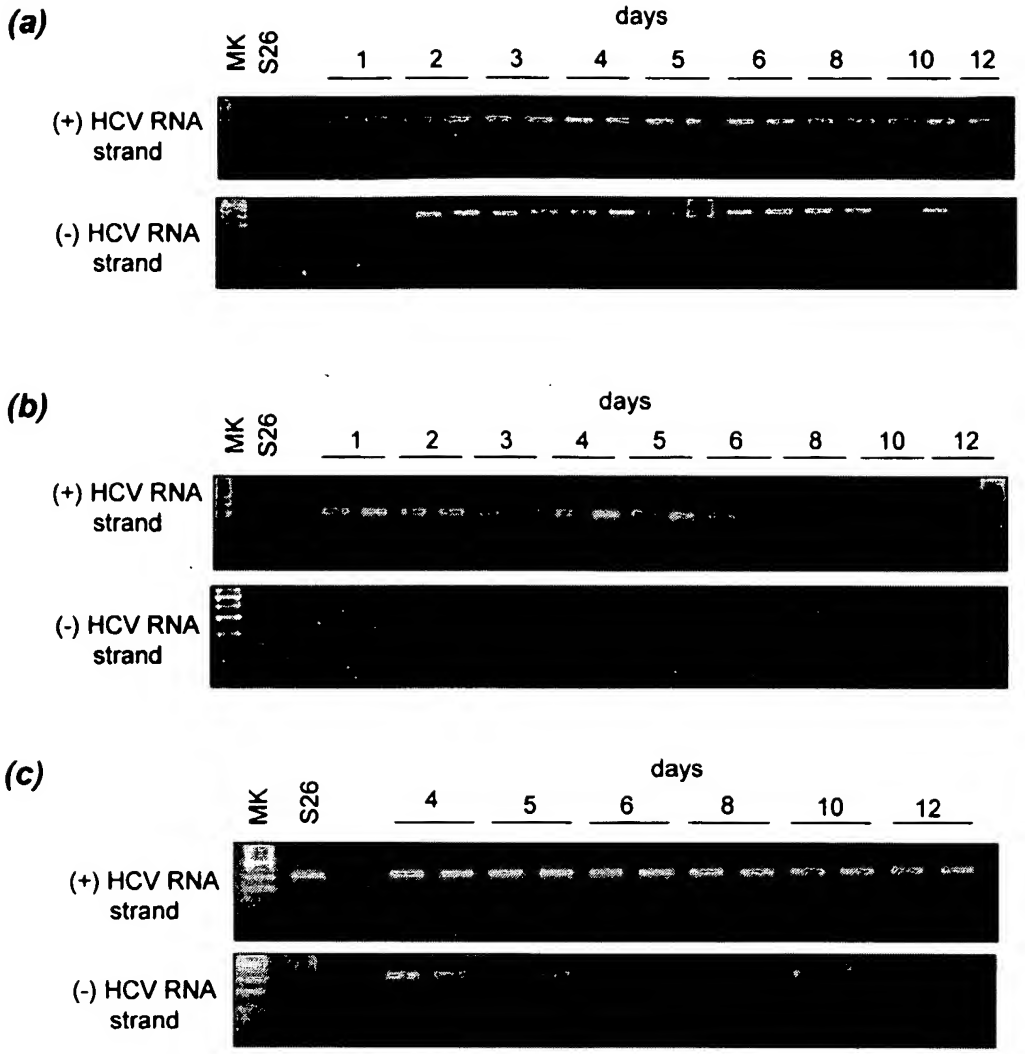

FIG. 2. Qualitative assay detection of positive- and negative-strand HCV RNA in a primary culture of healthy human hepatocytes infected in vitro with an HCV-positive serum and effect of IFN-α. The hepatocyte culture FT147, infected 3 days after plating by HCV-positive serum S26, is shown. Positive-strand (+) RNA but not negative-strand (-) RNA was present in the inoculum. (a) Primary hepatocyte culture in the absence of IFN-α. Positive-strand HCV RNA was detected with the qualitative strand-specific r*Tth* PCR assay from day 1 to the end of the culture (day 12), whereas negative-strand RNA was detected from days 2 to 10. (b) Culture in the presence of 5,000 U of IFN-α per ml. Positive-strand HCV RNA was detected from days 1 to 10, whereas negative-strand RNA was never detected. (c) Culture treated on day 3 with 5,000 U of IFN-α per ml. Positive-strand RNA was detected throughout the culture period, whereas negative-strand RNA was no longer detected after day 5. Similar patterns (not shown) were observed with the following cultures infected with the corresponding sera: FT141 and S23, FT143 and S34, FT144 and S27, FT154 and S23, FT155 and S20, and FT156 and S17. MK, molecular size standards.

FT168 infected with serum S34 (data not shown). Finally, neither positive- nor negative-strand HCV RNA was detected in culture supernatants by the sensitive qualitative assay.

In order to prove that HCV replicated in primary hepatocyte cultures, i.e., that HCV RdRp synthesized both negative- and positive-sense HCV RNA strands, we examined the accumulation of mutations on HCV genomes in five cultures. A 300-bp fragment located within the NS5A gene was chosen for this study. In all instances, nucleotide mutations accumulated on positive-strand HCV genomes during the culture period. Comparison of NS5A quasispecies sequences in the inoculum and after 8 days of culture (20 clones per time point) showed significantly higher between-sample genetic distances (calculated by pairwise comparison of NS5A quasispecies sequences in the inoculum versus the culture) than within-sample genetic distances (calculated by pairwise comparison of NS5A quasispecies sequences in the inoculum) (Fig. 4). In all instances, accumulation of synonymous mutations per synonymous site was significantly greater than accumulation of nonsynonymous mutations per nonsynonymous site (data not shown), indicating that the accumulation of mutations on HCV genomes in culture resulted from random nucleotide misincorporations by RdRp, in the absence of positive selection forces driving genetic evolution.

Together, these findings demonstrated unequivocally that HCV replicated in the primary cultures of healthy human

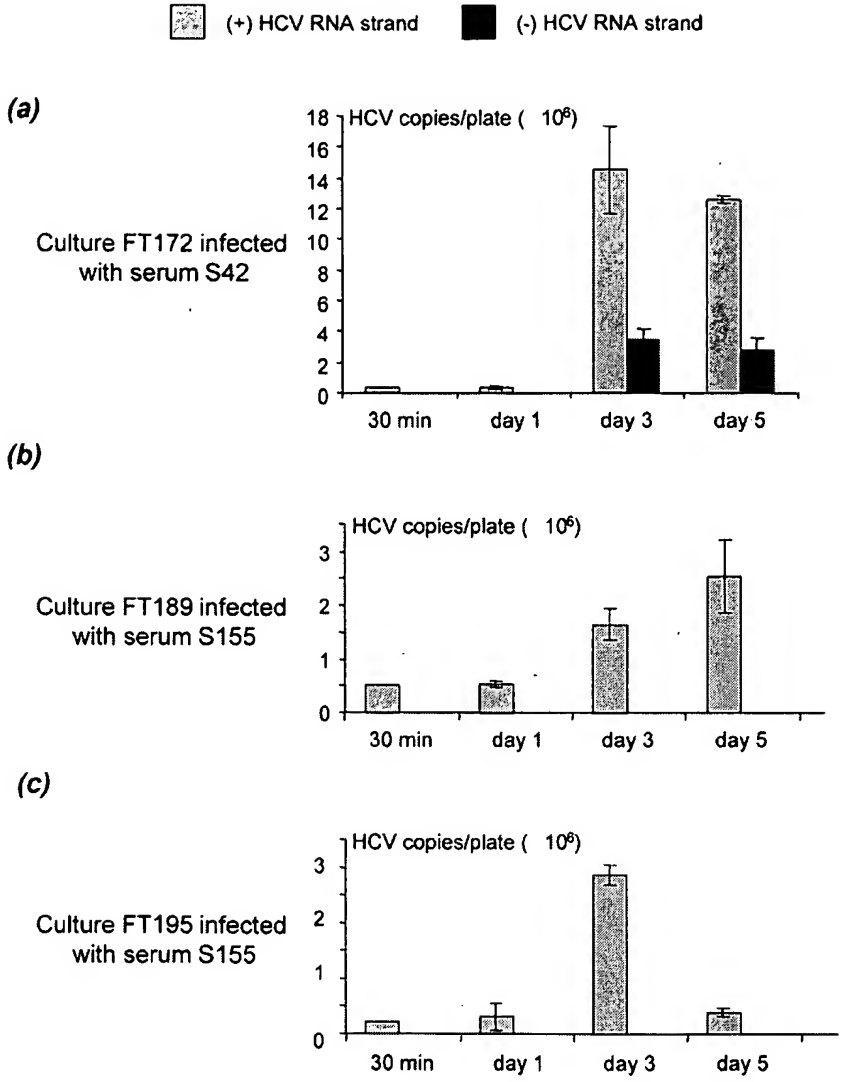

FIG. 3. Accumulation of positive- and negative-strand HCV RNA in hepatocyte cultures FT172 (a), FT189 (b), and FT195 (c), infected with sera S42, S155, and S155, respectively, as measured by the quantitative LightCycler real-time RT-PCR assay. The hepatocyte cultures were infected 3 days after plating. The cells were harvested 30 min and 1, 3, and 5 days after infection for positive-strand (gray) and negative-strand (black) HCV RNA quantification. The amounts of HCV RNA strands are shown as means ± SEMs of three determinations, expressed in numbers of HCV RNA copies per 2×10^6 cells, normalized to GAPDH mRNA. Similar results (not shown) were obtained with culture FT168 infected with serum S34.

hepatocytes as a result of viral RdRp function. Complete infectious virions did not appear to be secreted in the medium.

Primary cultures of healthy human hepatocytes exhibit a biological response to IFN-α. To determine whether primary hepatocyte cultures are equipped to respond appropriately to IFN-α stimulation, uninfected cultures were treated with 5,000 U of IFN-α per ml. We then extracted total cellular protein at various times from 0 to 24 h and analyzed the expression of IRF-1 and double-stranded PKR by Western blotting. These two proteins are encoded by two prototypic IFN-α-regulated genes: IRF-1 is a transcription factor induced as a primary response to IFN-α, while PKR induction is a secondary response, necessitating prior synthesis of IRF-1. IFN-α-stimulated Daudi cells were used as positive controls for these experiments. IFN-α induced the expression of both IRF-1 and PKR in cultured hepatocytes by factors of approximately 4 and 3, respectively (Fig. 5). As expected, IRF-1 expression preceded PKR expression by approximately 8 h. Similar experiments were carried out after 8 days of culture, with both uninfected and HCV-infected hepatocytes. Identical results were obtained, indicating that the response to IFN-α is maintained for more than a week and is not eliminated by HCV infection (Fig. 5).

IFN-α is not toxic for primary cultures of healthy human hepatocytes at the concentrations used in this study. As an effect of IFN-α on markers of intracellular HCV replication might merely reflect cytotoxicity rather than inhibition of viral RdRp, we studied IFN-α toxicity in our primary hepatocyte culture system. Phase-contrast microscopy revealed no signs of cellular toxicity. Furthermore, culture of hepatocytes from three different donors (FT154, FT155, and FT156), treated for 5 days with 5,000 U of IFN-α per ml, revealed no significant

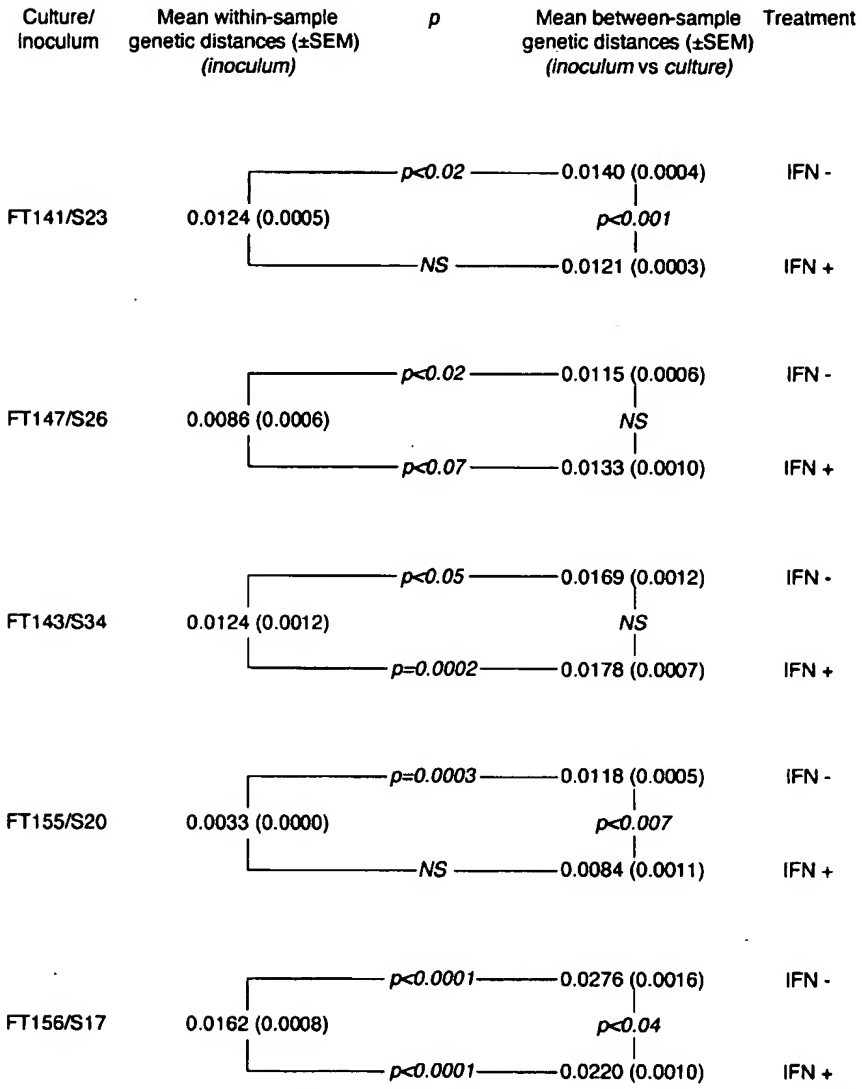

FIG. 4. Accumulation of nucleotide substitutions on HCV genomes during replication in five primary cultures of healthy human hepatocytes in the presence and absence of IFN-α. The accumulation of mutations on HCV genomes was assessed by comparing the mean ± SEM within-sample genetic distance (calculated by pairwise comparison of NS5A quasispecies sequences in the inoculum) with the mean ± SEM between-sample genetic distance (calculated by pairwise comparison of NS5A quasispecies sequences in the culture versus the inoculum). A significantly higher between-sample than within-sample genetic distance was interpreted as a significant accumulation of genomic mutations over time as a result of HCV replication in the culture; the lack of significant difference was interpreted as a lack of genetic evolution in the culture, reflecting inhibition of HCV replication. The between-sample genetic distances were also compared for each culture in the presence (+) and absence (-) of IFN-α. A significantly smaller between-sample genetic distance in the presence of IFN-α reflected reduced accumulation of mutations in the culture and was interpreted as an inhibition of HCV replication by IFN-α. NS, not significantly different (i.e., $P > 0.05$).

reduction in total de novo protein synthesis (not shown), a sensitive marker of cytotoxic stress in cultured hepatocytes (6).

IFN-α inhibits the expression of positive- and negative-sense HCV RNA strands in primary cultures of healthy human hepatocytes infected in vitro. We tested eight primary hepatocyte cultures infected in vitro for the effects of continuous incubation with 5,000 and 10,000 U of IFN-α per ml by means of the qualitative HCV RNA assay. In culture FT147 infected with serum S26 (Fig. 2b), the positive-sense RNA strand was detected from day 1 but disappeared after day 10 in the presence of IFN-α, whereas the negative-sense RNA strand remained undetectable throughout the culture period (i.e., until day 12). Similarly, the negative-sense RNA strand was never detected in any other IFN−α-treated culture (not shown). In contrast, when IFN-α treatment was started 3 days after HCV infection, the positive-sense RNA strand was detected throughout the culture period, whereas the negative-sense RNA strand was detected from infection through day 5 before disappearing (Fig. 2c).

The effect of increasing IFN-α concentrations (500 to 10,000 U/ml) on the detection of positive- and negative-sense HCV RNA strands was studied 5 or 8 days after infection. In cultures FT147 and FT161 (Fig. 6a), the negative-sense HCV RNA strand was never detected, whatever the IFN-α concentration

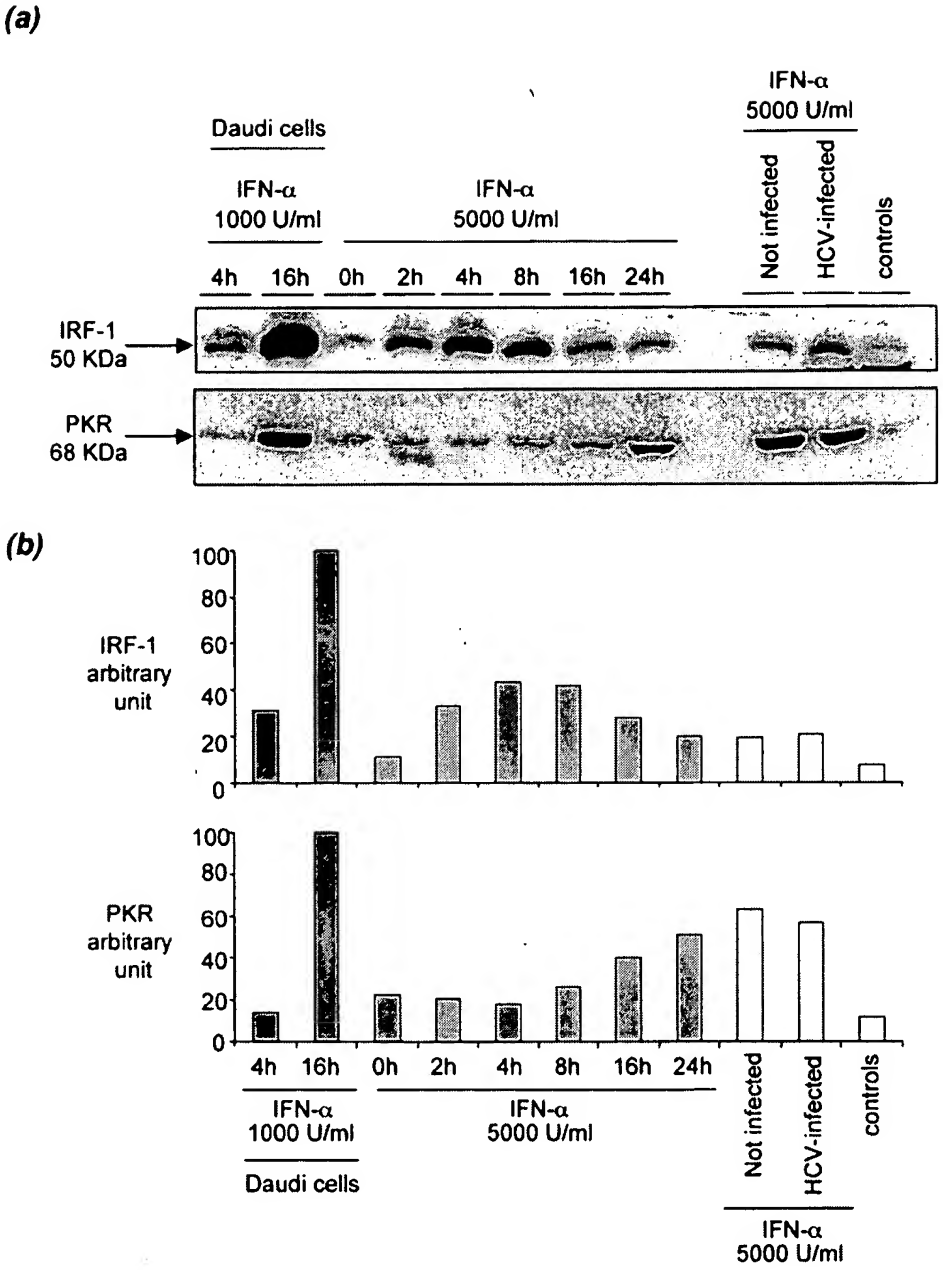

FIG. 5. Effects of IFN-α on IRF-1 and PKR expression in primary cultures of human hepatocytes. Immunoblot analysis was performed with anti-IRF-1 and anti-PKR antibodies after 8 days of culture in infected and noninfected primary hepatocytes treated with 5,000 U of IFN-α per ml and in Daudi cells treated with 1,000 U of IFN-α per ml used as positive controls. Cells not treated with IFN-α were used as controls. Immunoblot experimental results (a), together with their quantitative representation after National Institutes of Health image analysis (b) are shown. (b) (Left) Effect of 1,000 U of IFN-α per ml on Daudi cells harvested after 4 and 16 h of treatment. (Center) Effect of 5,000 U of IFN-α per ml on hepatocyte culture FT172 harvested after 0, 2, 4, 8, 16, and 24 h of treatment. (Right) Effect of HCV infection of the primary hepatocyte culture on the effect of IFN-α on IRF-1 and PKR expression. Similar results (not shown) were obtained in cultures FT164 and FT171.

used, whereas the positive-sense HCV RNA strand was always detected, even at the maximum IFN-α concentration used, i.e., 10,000 U/ml. Inhibition of viral replication in the culture was confirmed by using the less sensitive, but quantitative, real-time RT-PCR assay. Indeed, this assay did not detect positive-strand HCV RNA at IFN-α concentrations higher than 500 U/ml, meaning that the intracellular amount of positive-strand HCV RNA was below the detection cutoff level (Fig. 6b). Altogether, these results suggested potent concentration-dependent inhibition of positive-sense HCV RNA strand accumulation in response to IFN-α treatment.

IFN-α inhibits the accumulation of mutations on the HCV genome during replication in primary cultures of healthy human hepatocytes infected in vitro. In order to confirm that IFN-α inhibited HCV replication, we studied its effect on the accumulation of mutations on HCV genomes. In the absence

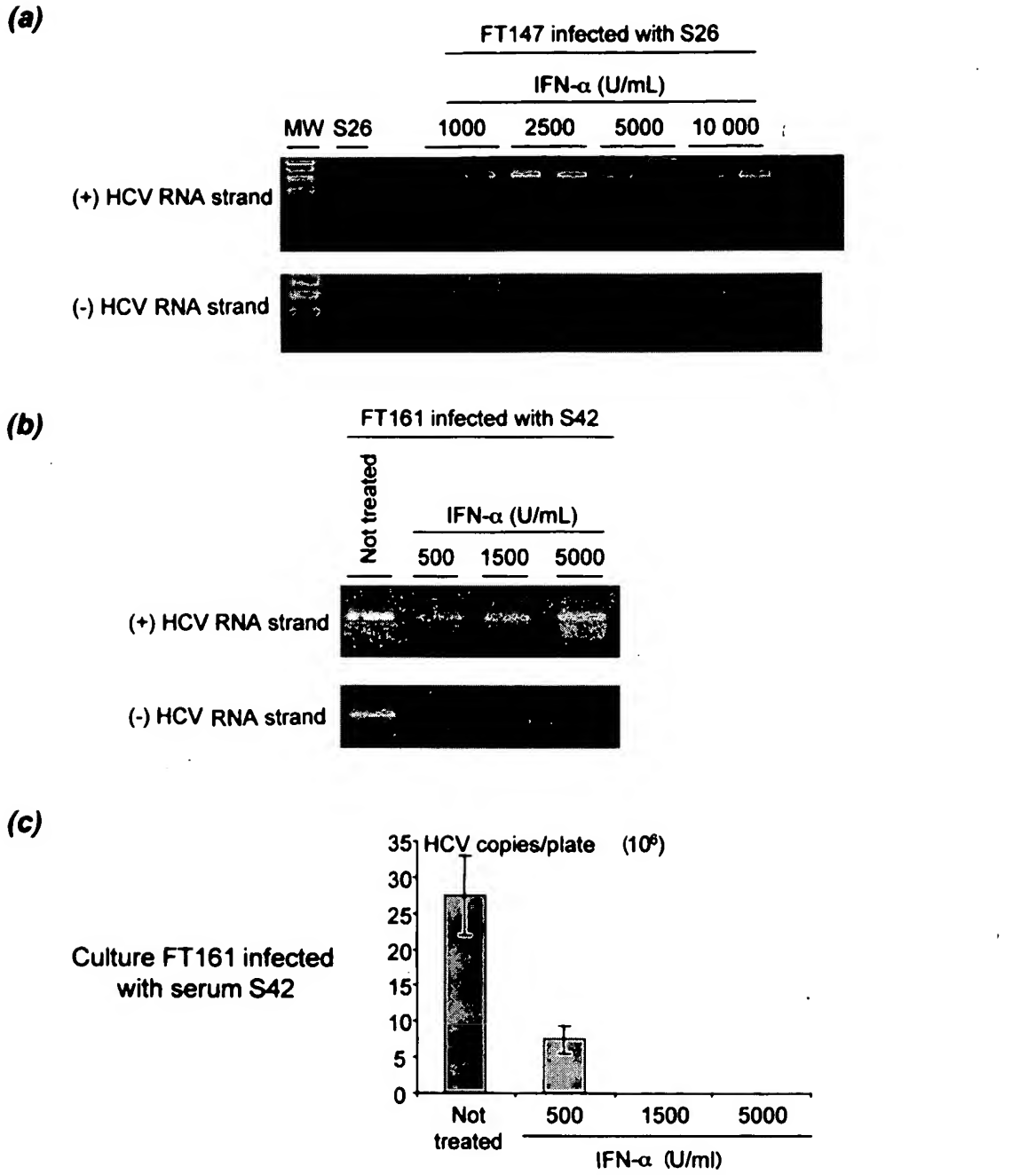

FIG. 6. Effect of increasing concentrations of IFN-α on the accumulation of positive- and negative-strand HCV RNA in primary hepatocyte cultures infected in vitro. Cultures FT147 (infected with serum S26) and FT161 (infected with serum S42) were treated for 5 and 8 days with IFN-α concentrations ranging from 1,000 to 10,000 U/ml and 500 to 5,000 U/ml, respectively. Qualitative detection of positive-sense (+) and negative-sense (-) HCV RNA strands is shown in cultures FT147 (a) and FT161 (b). In both instances, positive-strand HCV RNA was detected at all concentrations used, whereas the negative strand was never detected. MW, molecular size standards. (c) In culture FT161, LightCycler real-time RT-PCR quantitative analysis of the same extracts showed a reduction in the amount of positive-sense HCV RNA strand in the culture when the IFN-α concentration increased, suggesting IFN-α concentration-dependent inhibition of HCV replication in the culture. Similar results (not shown) were obtained with culture FT187 infected with serum S155.

of IFN-α, significant HCV genetic evolution was always observed, due to random accumulation of mutations (Fig. 4). In contrast, when 5,000 or 10,000 U of IFN-α per ml was added daily before testing for positive-strand RNA on day 8 of infection (i.e., several days before it otherwise became undetectable), no significant genetic evolution was observed in two of the five cultures tested (Fig. 4). In addition, the between-sample genetic distances (calculated by pairwise comparison of the quasispecies sequences in culture on day 8 versus that in the inoculum) were significantly lower in the presence of

IFN-α than in the absence of IFN-α in three of the five cultures, again suggesting that IFN-α significantly inhibited the accumulation of HCV genome mutations. No significant difference was seen in the remaining two cultures. In one culture, phylogenetic analysis followed by phylogenetic tree plotting showed a trend toward distinctive clustering of postculture and inoculum quasispecies sequences, respectively, in the absence of IFN-α; this clustering was abolished in the presence of IFN-α (data not shown). No such trend was clearly visible in the remaining four cultures.

DISCUSSION

This study shows that HCV can replicate in primary cultures of human hepatocytes infected in vitro, as a result of viral RdRp function. As previously described (7), in vitro infection resulted in the production of negative-sense HCV RNA strands, an HCV replication intermediate. In addition, real-time RT-PCR quantification showed a significant accumulation of positive-sense HCV RNA strands and, when present in sufficient amounts, of negative-sense HCV RNA strands during hepatocyte culture. Significant accumulation of random mutations on the HCV genome showed that the viral RdRp, an error-prone RNA polymerase with no proofreading activity, was responsible for the accumulation of positive-strand HCV RNA genomes during culture. The random accumulation of mutations in the region studied, in the absence of positive pressure toward amino acid changes, was not surprising in this in vitro culture system. However, the same region of the NS5A gene displays a similar pattern of genetic evolution in HCV-infected patients (33). As NS5A is part of the replication complex and is most likely involved in regulating RdRp function (13, 43), the conservation constraints on NS5A evolution occurring in vivo might also be present in our hepatocyte culture system.

The principal finding of this study is the effect of IFN-α on HCV replication in primary hepatocyte culture. IFN-α was recently shown to inhibit the replication of dengue virus, another member of the *Flaviviridae* family, in hepatoma cell lines infected in vitro (5). Mathematical modeling of HCV dynamics during human IFN-α treatment recently suggested that IFN-α blocks HCV virion production or release as a result of its direct, nonspecific antiviral effect (29, 30). The capacity of IFN-α to directly inhibit HCV replication in healthy human hepatocytes had not previously been demonstrated.

We show that IFN-α blocks HCV genome synthesis by the HCV RdRp in cultured healthy human hepatocytes in a concentration-dependent manner. This effect is probably mediated by IFN-α-induced cellular pathways supporting nonspecific antiviral actions. Indeed, we observed the following. (i) IFN-α-induced genes were expressed in primary hepatocyte cultures treated with IFN-α, and their expression was not altered by HCV infection. (ii) Expression of negative-strand HCV RNA was always suppressed in IFN-α-treated cultures. (iii) IFN-α significantly inhibited the accumulation of mutations on the HCV genome in three of five hepatocyte cultures. The concentrations of IFN-α used here were relatively high (500 to 10,000 U/ml), but we showed that IFN-α toxicity could not explain the inhibitory effect on HCV replication. An earlier report suggested that IFN-α could act by inhibiting de novo infection of hepatocytes (48). If IFN-α effectively prevents HCV entry into hepatocytes in vivo, this effect would probably be mediated by IFN-α-induced humoral responses (neutralizing antibodies), which are not present in hepatocyte cultures. We did not examine whether IFN-α could affect virus entry in our model, in addition to inhibiting viral replication. It is conceivable that the reduction of positive-strand HCV RNA accumulation in IFN-α-treated cells could be enhanced by receptor down-regulation or by decreased internalization or membrane fusion.

Interestingly, despite the disappearance of negative-strand HCV RNA from all IFN-α-treated cultures, positive-strand RNA always persisted for several days, suggesting that the kinetics of HCV RNA strands in cell culture differ from those in the peripheral circulation. This finding was not surprising, because most of the mechanisms governing viral clearance are absent in vitro, especially when only intracellular HCV RNA is concerned, whereas the estimated mean half-life of free HCV virions is only 2.7 h in vivo (30). In contrast, the apparent lack of IFN-α inhibition of mutation accumulation on HCV genomes in two cultures, despite a clear effect on negative-strand HCV RNA production, was surprising. The inhibitory effect of IFN-α may have been weaker in these two cultures, permitting low-level viral replication, while negative-strand HCV RNA was undetectable, even with our sensitive qualitative strand-specific HCV RNA assay. Such variability in the effect of IFN-α might be explained by partial hepatocyte resistance to IFN-α stimulation or by partial viral resistance to IFN-α, possibly mediated by viral proteins inhibiting antiviral effectors induced by IFN-α (32). It is noteworthy that all the cultures were infected with HCV genotype 1, a genotype that displays various levels of IFN-α sensitivity based on initial IFN-α blocking efficacy (16, 30), possibly owing to differences in the sequences of viral proteins and, thus, in their structure and function. Unfortunately, data on early viral dynamics during IFN-α therapy in the patients whose blood samples were used for in vitro infection are not available to confirm this hypothesis.

In conclusion, we show that primary cultures of healthy human hepatocytes can be infected in vitro by HCV and support its sustained replication. We further show that IFN-α is able to block HCV replication in this culture model, which is close to the HCV-infected human liver. These results strengthen the hypothesis that IFN-α acts primarily through its nonspecific antiviral effects and suggest that primary cultures of human hepatocytes may provide a good model to study intrinsic HCV resistance to IFN-α. However, clearance of infected cells resulting from IFN-α-modulated immune responses probably plays a major role in permanent HCV RNA clearance during therapy (16, 30), emphasizing the need for both in vitro and in vivo studies to understand IFN-α treatment failure in HCV-infected patients.

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Valerie Castet and Chantal Fournier contributed equally to this work.

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Replication of Subgenomic Hepatitis C Virus RNAs in a Hepatoma Cell Line

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An estimated 170 million persons worldwide are infected with hepatitis C virus (HCV), a major cause of chronic liver disease. Despite increasing knowledge of genome structure and individual viral proteins, studies on virus replication and pathogenesis have been hampered by the lack of reliable and efficient cell culture systems. A full-length consensus genome was cloned from viral RNA isolated from an infected human liver and used to construct subgenomic selectable replicons. Upon transfection into a human hepatoma cell line, these RNAs were found to replicate to high levels, permitting metabolic radiolabeling of viral RNA and proteins. This work defines the structure of HCV replicons functional in cell culture and provides the basis for a long-sought cellular system that should allow detailed molecular studies of HCV and the development of antiviral drugs.

HCV is a plus (+) strand RNA virus that causes acute and chronic liver diseases (*1*). Although the acute phase of infection is usually associated with mild symptoms, most patients fail to clear the virus and contract persistent infection that frequently leads to chronic liver disease, including cirrhosis and hepatocellular carcinoma. Given the high prevalence of the virus, HCV has become a focus of intensive research.

Originally cloned in 1989 (*2*), the viral genome is now well characterized. It has a length of ~9.6 kb and its single, long open reading frame (ORF) encodes a ~3000–amino acid polyprotein (*3*) (Fig. 1A). The ORF is flanked at the 5′ end by a nontranslated region (NTR) that functions as an internal ribosome entry site (IRES) and at the 3′ end by a highly conserved sequence essential for genome replication (*4*). The structural proteins are in the NH_2-terminal region of the polyprotein and the nonstructural proteins (NS) 2 to 5B in the remainder. By analogy to related +strand RNA viruses, replication occurs by means of a minus (−) strand RNA intermediate and is catalyzed by the NS proteins forming most likely a cytoplasmic membrane–associated replicase complex.

Despite the availability of cloned infectious genomes (*5*), molecular studies of HCV replication and the development of antiviral drugs have been hampered by the low efficiencies of currently available cell culture systems and by the fact that the only animal model is the chimpanzee. Thus, to date, research on HCV replication has depended largely on the infection of cell lines or primary cell cultures with sera and the detection of viral replicative intermediates with the highly sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) (*6*).

To overcome these limitations, we established an efficient cell culture system that is based on the transfection of cloned viral consensus genome sequences. Owing to the high amount of HCV RNA, we used as starting material for cloning total liver RNA isolated from a chronically infected patient who had undergone liver transplantation (*7*). Using long-distance RT-PCR, we amplified the complete ORF in two overlapping fragments. Several clones of each fragment were analyzed, and an isolate-specific consensus sequence was established, which belongs to the worldwide distributed genotype 1b. The 5′ and 3′ NTRs were amplified separately by standard RT-PCR and were assembled with the reconstituted consensus ORF. A 5′-flanking T7 RNA polymerase promoter and an engineered restriction site at the 3′ end allowed for production of run-off RNA transcripts with authentic 5′- and 3′-terminal sequences (*8*). As a negative control for all transfection experiments for each parental construct, a defective genome was generated carrying an in-frame 10–amino acid deletion (Δ) encompassing the NS5B RNA polymerase active site.

We initially transfected various cell lines and primary human hepatocytes with in vitro transcripts corresponding to the cloned full-length genome or the deletion mutant. We monitored RNA replication by comparing the amounts of +strand RNA detected by RT-PCR in cells transfected with the parental or the defective genome (*9*). In no case was a significant difference found between the genomes, suggesting that no replication occurred.

The failure of these experiments might be attributable to errors in the cloned genome, low transfection efficiencies, or cytopathogenicity of HCV, which would lead to a selective loss or growth disadvantage of cells supporting virus replication. Alternatively, the cell lines used might be nonpermissive or support only low levels of viral RNA replication not detectable with our method. To overcome some of these restrictions, we constructed selectable replicons that transduced neomycin (G418) resistance only to those cells that support HCV replication. Based on recent results with flavi- (*10*) and pestiviruses (*11*) and on mapping of the HCV-IRES, we generated bicistronic constructs (*12*) (Fig.

Table 1. G418-resistant cell clones obtained after transfection of Huh-7 cells with in vitro–transcribed HCV replicon RNAs. The number of viable cell clones after subpassage of clones obtained in experiment 1 is given in the right column.

Construct	Experiment			Subpassages (exp. 1)
	1	2	3	
I_{377}neo/NS2-3′/wt	12	20	38	1
I_{377}neo/NS2-3′/Δ	0	3	8	0
I_{377}neo/NS3-3′/wt	20	>60	40	2
I_{377}neo/NS3-3′/Δ	2	8	18	0
I_{389}neo/NS2-3′/wt	6	20	25	1
I_{389}neo/NS2-3′/Δ	1	10	4	1
I_{389}neo/NS3-3′/wt	30	15	17	5
I_{389}neo/NS3-3′/Δ	1	2	6	0

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1A). These were composed of two variants of the HCV-IRES [nucleotides (nt) 1 to 377 or 1 to 389], the neomycin phosphotransferase (*neo*) gene, the IRES of the encephalomyocarditis virus, which directs translation of HCV sequences from NS2 or NS3 up to NS5B, and the 3′ NTR. Therefore, these replicons were designated I_{377}/NS2-3′ (or I_{377}/NS3-3′) and I_{389}/NS2-3′ (or I_{389}/NS3-3′). In vitro transcripts derived from these constructs were transfected in parallel with the analogous mutants carrying the in-frame deletion of the NS5B polymerase active site (Δ constructs). Particular care was taken to remove template DNA, which might otherwise integrate into transfected cells and confer G418 resistance independent of HCV replication (*13*). In three separate experiments performed with the human hepatoma cell line Huh-7 (*14*), we observed a clear difference in the number of resistant cell clones between the parental (wild-type, wt) and the defective (Δ) replicons (Table 1) (*15*). Cell clones obtained in the first experiment were isolated and subpassaged. Most of the cells died during this procedure and ultimately we obtained nine clones derived from transfection with parental replicons and one clone derived from transfections with a defective NS2-3′ RNA (clone 8-1). With the exception of a reduced doubling time, no consistent difference was found between these nine cell clones and clone 8-1 or the parental Huh-7 cell line.

The main criteria for functional replicons are the formation of viral RNAs of correct size and the absence of (integrated) plasmid DNA that could confer G418 resistance. To detect HCV RNAs in these cells, we isolated total RNAs and analyzed a sample on Northern blots using a +strand-specific RNA probe (Fig. 1B). With the exception of clone 8-1, homogeneous RNAs of correct lengths (~ 8640 nt for the NS2-3′ and ~ 7970 nt for the NS3-3′ replicon) were detected, suggesting that functional replicons conferred the G418 resistance. Although the amount of HCV RNA detected in these clones was variable during passages, the highest amount was consistently obtained with cell clones 5-5, 5-15, and 9-13 (lanes 6, 8, and 11). To exclude the possibility that resistance was due to plasmid DNA integrated into the host cell genome and transcribed under control of a cellular promoter, we analyzed DNA of each clone by *neo*-specific PCR (Fig. 1C). With the exception of cell clones 7-3 and 8-1 (lanes 3 and 12), no *neo*-DNA was detected, confirming that G418 resistance of most clones was conferred by HCV RNA replicons. Irrespective of these results, generation of HCV RNAs of the correct size from integrated plasmid DNAs is highly unlikely, because the plasmid used for in vitro transcription contained neither a eukaryotic promoter nor a polyadenylation signal. Therefore, in the case of clone 7-3, resistance most likely was mediated both by the replicon and integrated *neo*

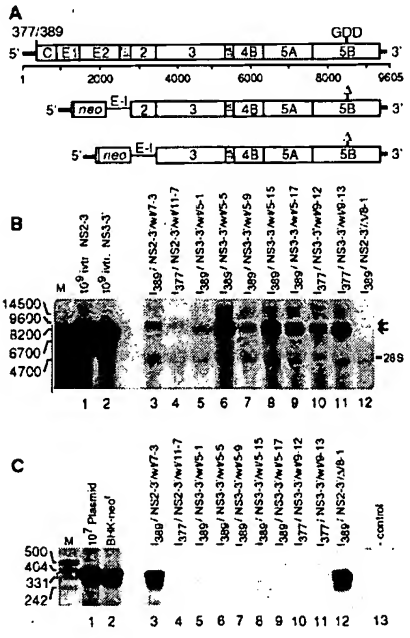

Fig. 1. Structure of the HCV subgenomic replicons and detection of viral RNA replication in transfected Huh-7 cells. **(A)** (Top) Schematic of the HCV genome, indicating the location of cleavage products within the polyprotein and the 5′ and 3′ NTRs (thick lines). The positions of the 3′ borders of the HCV-IRES selected for construction and the "GDD active site" of the NS5B RNA polymerase are shown above. Numbers below the genome refer to the nucleotide positions of our consensus isolate. The structures of the selectable replicons composed of the 5′ HCV-IRES, the *neo* gene, the EMCV-IRES (E-I), and HCV sequences from NS2 or NS3 up to the authentic 3′ end are given below. Δ indicates the position of the 10–amino acids deletion in the NS5B polymerase (amino acids 2732 to 2741 of the polyprotein). **(B)** Detection of +strand HCV RNA in subpassaged Huh-7 cell clones. Total RNA was isolated from the cells (*20*), and 10 μg of RNA corresponding to 5×10^5 cells was analyzed by denaturing agarose gel electrophoresis. Replicon RNA was detected by Northern blot with a radiolabeled RNA probe complementary to the *neo* gene and the HCV-IRES. In vitro transcripts (10^9) (ivtr.) corresponding to the parental I_{389}-replicons were analyzed in parallel (lanes 1 and 2). Arrows point to HCV RNAs. Lane M, positions of RNA size markers (in nucleotides); the position of the 28*S* ribosomal RNA is indicated on the right. The RNA marker fragments contain HCV sequences and therefore hybridize with the RNA probe. **(C)** Absence of integrated replicon DNA in most selected cell clones. DNA was isolated from Huh-7 clones with nucleo-spin columns (Macherey-Nagel, Düren, Germany) and subsequently treated with ribonuclease A for 1 hour. After phenol-chloroform extraction and ethanol precipitation, 1 μg of DNA corresponding to 4×10^4 to 8×10^4 cells was analyzed by PCR with *neo*-specific primers (5′-TCAAGACCGACCTGTCCGGTGCCC-3′ and 5′-CTTGAGCCTGGCGAACAGTTCGGC-3′). Amplified fragments were analyzed by Southern (DNA) blot with a digoxigenin-labeled DNA probe corresponding to the *neo* gene. As a positive control, PCR was performed with 10^7 plasmid molecules or 1 μg of DNA from a BHK cell line stably transfected with the *neo* gene (lanes 1 and 2), and as a negative control PCR was performed without a DNA template (lane 13). Lane M, molecular size markers (in base pairs).

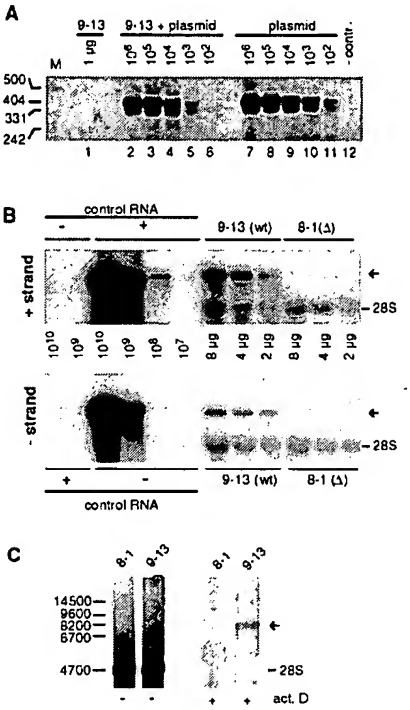

Fig. 2. Characterization of cell clone 9-13, which harbors the NS3-3′ replicon RNA. **(A)** Exclusion of *neo* DNA was done by PCR and Southern blot as in Fig. 1C with 1 μg of DNA (lane 1). Sensitivity was determined by using 10^6 to 10^2 plasmid molecules (I_{377}/NS3-3′/wt) either directly for PCR (lanes 7 to 11) or after addition of 1 μg of 9-13 DNA (lanes 2 to 6). As a negative control, PCR was performed without DNA (lane 12). **(B)** Quantification of HCV + and −strand RNA. Eight, 4, or 2 μg of total RNA isolated from cell clones 9-13 and 8-1 were analyzed by Northern blot in parallel with analogous in vitro transcripts of given polarity (control RNA). +Strand RNA (upper panel) was detected with an RNA probe complementary to the *neo* gene and the HCV IRES, and −strand RNA (lower panel) was detected with an RNA probe complementary to the NS3 sequence. Arrows indicate the positions of replicon RNAs. **(C)** Replication of HCV RNA is resistant to actinomycin D. About 5×10^5 cells of clones 9-13 and 8-1 were incubated with 100 μCi of [^{3}H]uridine for 16 hours in the absence (−) or presence (+) of actinomycin D (act. D, 4 μg/ml). After labeling, total RNA was prepared and analyzed by denaturing agarose gel electrophoresis. Only one-tenth of total RNA is shown in the first two lanes. Radiolabeled RNAs were visualized with a BAS-2500 Bio-Imager (Fuji).

DNA sequences, whereas resistance of cell clone 8-1 was conferred exclusively by integrated plasmid DNA.

To confirm that G418 resistance was mediated by autonomously replicating HCV RNAs, we chose cell clone 9-13 for further analysis, because it contained high amounts of HCV RNA, and used clone 8-1 throughout as a negative control. To rule out the presence of *neo*-DNA in clone 9-13 with high stringency, we performed a PCR assay that allowed detection of <0.02 *neo* copies per cell (Fig. 2A). Even with this level of sensitivity, no plasmid DNA was found. To calculate the amounts of HCV + and −strand RNAs in these cells, we analyzed serial dilutions of total RNA on Northern blots using strand-specific radiolabeled RNA probes (Fig. 2B). About 10^8 +strand RNA copies per microgram of total RNA were detected; this corresponds to 1000 to 5000 molecules per cell. A 5- to 10-fold lower amount of −strand RNA was detected, consistent with the notion that −strand RNA is the replicative intermediate serving as template for synthesis of excess +strand molecules. Because this reaction is carried out by the NS5B RNA-dependent RNA polymerase, generation of HCV RNAs should be resistant to actinomycin D, an antibiotic that selectively inhibits RNA synthesis from DNA but not RNA templates. To test this hypothesis, we incubated cells with [^{3}H]uridine in the presence of actinomycin D and analyzed the radiolabeled RNAs (Fig. 2C). In agreement with the inhibitor profile of the NS5B polymerase (*16*), replication of HCV RNA was not affected by actinomycin D, whereas synthesis of cellular RNAs was blocked.

For analysis, the viral proteins were radiolabeled with ^{35}S-methionine and ^{35}S-cysteine and isolated by immunoprecipitation. All HCV proteins were detected, and they corresponded in size to the proteins observed after transient expression of the same replicon construct in naïve Huh-7 cells (Fig. 3A). Immunostaining with NS3- and NS5A-specific antisera revealed that NS3 and NS5A localized almost exclusively to the cytoplasm, although there was a small amount of NS5A staining in the nucleus (Fig. 3B). This predominant cytoplasmic localization of viral antigens provides strong evidence that HCV RNA replicates in the cytoplasm, as is the case for most RNA viruses.

Our data define the structures of selectable HCV RNAs replicating autonomously and to high levels in a human hepatoma cell line. The fact that the structural proteins and NS2 are not required for replication emphasizes the close evolutionary relationship between HCV and the animal pathogenic pestiviruses, for which analogous RNAs have been described (*11*). Although replication of these RNAs has not been quantified yet at a per cell basis, pestiviruses appear to replicate more efficiently, yielding titers of ~1.5 × 10^4 genomes per cell in the acute phase of infection (*17*). The ~10-fold lower value we found with the HCV replicons might reflect intrinsically lower HCV replication or the fact that the cell clones we established resemble a persistent and not an acute infection.

The low number of cell clones obtained may indicate that only a few cells in the culture support HCV RNA replication, or that a level of replication required for G418 resistance was reached in only a few cells. Alternatively, high-level replication may reflect an adaptation of the replicon to the host cell. As such adaptation would require one or several mutations, formation of an adapted replicon would be rare. However, this possibility is unlikely for two reasons: first, sequence analysis of several replicons recloned from two different cell clones did not reveal consistent mutations (*18*); second, upon serial passage of the replicons in naïve Huh-7 cells, we did not observe a significant increase of the number of colonies (*19*). Thus, the low efficiency most likely is attributable to particular host cell conditions or factors present in only a few cells.

The replicons described in this study may allow a detailed analysis of HCV replication, pathogenesis, and evolution in cell culture. In principle, viral RNAs can be generated in unlimited quantities, and the viral genome can be manipulated for genetic analyses of HCV functions that are essential for replication. Functional replicons also provide a cell-based assay system for the evaluation of antiviral drugs.

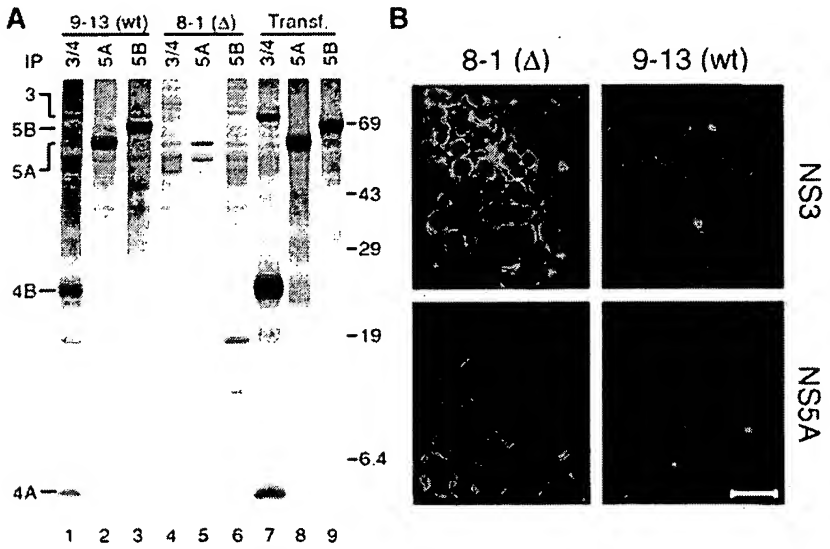

Fig. 3. Detection of HCV antigens in cell clone 9-13. (**A**) 9-13 (wt) and 8-1 (Δ) cells were incubated with protein labeling mixture (NEN Life Science) for 16 hours, and HCV proteins were isolated from cell lysates by immunoprecipitation (IP) under nondenaturing conditions (*21*) with antisera specified above the lanes. Immunocomplexes were analyzed by Tricine SDS–polyacrylamide gel electrophoresis and visualized by autoradiography. To obtain authentic size markers, the homologous replicon construct (I_{377}/NS3-3′/wt) was transiently expressed with the vaccinia virus T7-hybrid system (*22*) in Huh-7 cells and processed in parallel (lanes 7 to 9). Positions of HCV-proteins are given on the left, and molecular size standards (kilodaltons) on the right. The NS3/4-specific antiserum preferentially reacts with NS4A and NS4B. (**B**) Subcellular localization of HCV antigens as determined by immunofluorescence. Twenty-four hours after seeding on cover slips, 9-13 (wt) and 8-1 (Δ) cells were fixed with methanol-acetone, incubated with polyclonal NS3- or NS5A-specific antisera, and bound antibody was detected with a fluorescein isothiocyanate–conjugated antibody to rabbit immunoglobulin G. Cells were counterstained with Evans blue. Bar, 25 μm.

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7. Total RNA was isolated from explanted liver (~100

mg) (20), and 1 μg was used for reverse transcription with primers A6103 (GCTATCAGCCGGTTCATCCACTGC) or A9413 (CAGGATGGCCTATTGGCCTGGAG) and the Expand Reverse Transcriptase System (Boehringer Mannheim, Germany). PCR was performed with the Expand Long Template System (Boehringer Mannheim) in buffer containing 2% dimethylsulfoxide. After 1 hour at 42°C, one-eighth of the mixture was used for the first PCR with primers A6103 and S59 (TGTCTTCACGCAGAAAGCGTCTAG) or A9413 and S4542 (GATGAGCTCGCCGCGAAGCTGTCC). After 40 cycles, one-tenth was used for the second PCR with primers S59 and A4919 (AGCACAGCCCGCGTCATAGCACTCG) or S4542 and A9386 (TTAGCTCCCCGTTCATCGGTTGG). After 30 cycles, the PCR products were purified by preparative agarose gel electrophoresis, and eluted fragments were ligated into vector pCR2.1 (Invitrogen) or pBSK II (Stratagene). Four clones of each fragment were analyzed and a consensus sequence was established. To resolve ambiguities, we amplified shorter PCR fragments covering the corresponding region and sequenced multiple clones. The 3′ NTR was obtained by conventional PCR with an antisense primer covering the last 24 nt of the genome (4). The authentic 5′ NTR downstream of the T7 promoter was generated by PCR with an oligonucleotide corresponding to a truncated T7 promoter (TAATACGACTCACTATAG) and the first 88 nt of HCV and a plasmid carrying one of the 5′ fragments of the genome. The complete genome was assembled from subgenomic fragments carrying the least numbers of nonconsensus nucleotide changes and inserted into a modified pBR322 vector. Nonconsensus changes were removed by site-directed mutagenesis. To generate run-off transcripts with an authentic 3′ end, we modified the 3′ NTR of our isolate (terminating with TGT) to match the sequence of genotype 3 [clone WS; A. A. Kolykhalov, S. M. Feinstone, C. M. Rice, *J. Virol.* **70**, 3363 (1996)] terminating with AGT, which allowed us to introduce a recognition sequence for the restriction enzyme Sca I (AGTACT) at the end of the 3′ NTR. A guanine was replaced with an adenine nucleotide at position 8180 of the genome to remove an internal Sca I site. After assembly of the full-length genome with appropriate 5′ and 3′ NTRs, the complete HCV sequence [European Molecular Biology Laboratory (EMBL) accession number AJ238799] was verified.

8. Plasmid DNA was linearized with Sca I and used for in vitro transcription reactions containing 80 mM Hepes (pH 7.5), 12.5 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol, 2 mM of each nucleoside triphosphate, RNasin (1 U/ml), DNA template (50 μg/ml), and T7 RNA polymerase (~2 U/μl). To increase the yields, after 2 hours at 37°C an extra 1 U of T7 RNA polymerase was added per microliter, and the reaction was incubated for an additional 2 hours. DNA was removed by extraction with acid phenol [W. Kedzierski and J. C. Porter, *BioTechniques* **10**, 210 (1991)] and treatment with 2 U of deoxyribonuclease (DNase) per microgram of DNA for 60 min at 37°C. RNA was purified and analyzed by denaturing agarose gel electrophoresis.
9. Purified in vitro transcripts corresponding to the parental or the inactivated HCV genome were used for transfection of human hepatoma cell lines and primary human hepatocytes. Cell lines were maintained in a medium as described [B. J. Yoo *et al.*, *J. Virol.* **69**, 32 (1995)] and passaged once a week. Total RNA was prepared from transfected cells, and serial dilutions were used for RT-PCR amplification of the 5′ NTR or an NS5B sequence covering the 10–amino acid deletion. This allowed discrimination between the parental and the inactivated genome carrying the in-frame deletion. We monitored RNA replication by comparing the amounts of HCV RNA found in cells transfected with the wild-type or the inactivated genome. Input RNA was detected for up to three passages, with similar amounts seen for both genomes.
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18. As will be reported elsewhere (V. Lohmann and R. Bartenschlager, in preparation), we recloned HCV replicons from 1 μg of total RNA by RT-PCR using primers S59 and A9413 (7). For amplification of 5′ and 3′ NTRs, we used an RNA ligation approach before PCR. Among 10 sequenced replicons, no converging mutations were found. Each replicon contained 6 to 12 amino acid substitutions scattered throughout the HCV ORF. The NTRs were highly conserved, and only sporadic nucleotide changes were observed.
19. HCV RNA contained in total RNA of cell clones 5-15 and 9-13 was quantified by Northern blot, and 20 μg of total RNA were used for transfection (15). An equivalent number of in vitro–transcribed replicon molecules was supplemented with total RNA from naïve Huh-7 cells to the same concentration and transfected in parallel. Cotransfection of a construct directing the expression of firefly luciferase was used to correct for transfection efficiency. No significant difference in the number of G418-resistant colonies was found between total RNA isolated from the two cell clones and the in vitro RNA mixture.
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Positive Selection of Natural Autoreactive B Cells

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Lymphocyte development is critically influenced by self-antigens. T cells are subject to both positive and negative selection, depending on their degree of self-reactivity. Although B cells are subject to negative selection, it has been difficult to test whether self-antigen plays any positive role in B cell development. A murine model system of naturally generated autoreactive B cells with a germ line gene–encoded specificity for the Thy-1 (CD90) glycoprotein was developed, in which the presence of self-antigen promotes B cell accumulation and serum autoantibody secretion. Thus, B cells can be subject to positive selection, generated, and maintained on the basis of their autoreactivity.

Although it is widely accepted that B cells with self-reactivity are deleted or rendered functionally inactive (*1*), autoantibodies can be found in the serum of healthy animals, referred to as "natural autoantibodies," in an apparent paradox to the clonal tolerance theory (*2, 3*). In contrast with disease-associated hypermutated immunoglobulin G (IgG) antibodies, these natural autoantibodies are predominantly IgM, encoded by mostly unmutated germ line variable (V) region genes, and are independent of T cell help for secretion. Natural autoantibody constitutes a large fraction of serum Ig, and the B cells that produce natural autoantibodies frequently express CD5, a phenotype rare in spleen, but more common in the peritoneal cavity of mice (*4*,

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ORIGINAL REPORT

Pegylated Arginine Deiminase Treatment of Patients With Unresectable Hepatocellular Carcinoma: Results From Phase I/II Studies

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ABSTRACT

Purpose
Recently, we reported that a large number of human hepatocellular cancer (HCC) cell lines were auxotrophic for arginine. Here we report the results obtained with the amino acid–degrading enzyme arginine deiminase (ADI) conjugated to polyethylene glycol (ADI-SS PEG 20,000 mw) as a means of lowering plasma arginine to treat HCC. The study was a cohort dose-escalation phase I/II study.

Patients and Methods
Pharmacodynamic studies indicated an ADI-SS PEG 20,000 mw dose level of 160 U/m^2 was sufficient to lower plasma arginine from a resting level of approximately 130 μmol/L to below the level of detection (< 2 μmol/L) for more than 7 days, a dose later defined as the optimal biologic dose. All patients were to receive three cycles at the optimum biologic dose.

Results
This therapy was well tolerated, even in patients who had no detectable plasma arginine for 3 continuous months of therapy. Of the 19 patients enrolled, two had a complete response, seven had a partial response, seven had stable disease, and three had progressive disease. The median survival for the 19 patients enrolled on this study was 410 days, with four patients still alive at present (> 680 days).

Conclusion
Elimination of all detectable plasma arginine in patients with HCC was well tolerated and seemed to be effective in the treatment of some patients with HCC. Further testing of ADI-SS PEG 20,000 mw in a larger population of individuals with HCC as well as other human tumors auxotrophic for arginine is warranted.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world.[1-4] The international yearly incidence is approximately 1 million cases. In the United States, approximately 20,000 new cases are diagnosed annually, with more than 18,700 deaths annually. Thus the incidence and mortality rates are almost equal.

Current therapy for HCC is inadequate.[1-6] Fewer than 10% of HCC patients are candidates for surgical resection or transplantation. Even those who undergo resection have a poor long-term prognosis, as most relapse within 2 to 3 years with hepatic and/or systemic metastatic disease.[3,4,6] Systemic chemotherapy, either as a single agent or in combination, has not resulted in prolonged long-term survival rates. Hepatic arterial infusion of chemotherapy has yielded some increased long-term response rates, but this therapy as well as radiotherapy and hepatic arterial embolization have limited and most often only palliative benefit. Despite all forms of current treatment, most patients die within 1 year of diagnosis. Median life expectancy of pa-

ADI
ARG → CIT
ASS

tients with nonresectable disease has been historically reported as 1.4 to 3 months.[3,4,7-10]

Arginine is one of the nonessential amino acids for humans.[11] Normal cells and tissues synthesize arginine from citrulline in two steps using the urea cycle enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase. Some human cancers do not express ASS and thus are unable to synthesize arginine from citrulline. Therefore, it has been suggested that an arginine-degrading enzyme may prove effective in the eradication or control of arginine auxotrophic cancers.[12-18] We have shown that all available human HCC cell lines from the American Type Culture Collection do not express ASS and thus are auxothrophic for arginine.[17]

Various HCC cell lines have been shown to be killed in vitro and in vivo by arginine deiminase (ADI).[14,17] However, this form of therapy has two major disadvantages. First, ADI is not produced by mammals and must be derived from microbes. As a consequence, nascent ADI is strongly antigenic in mammals. Second, ADI has a short circulating half-life in mammals (approximately 5 hours) and must be administered in large daily doses to inhibit tumors implanted into mice.[13,19] ADI was formulated with polyethylene glycol (PEG) to produce ADI-SS PEG 20,000 mw.[19] This formulation of ADI is less antigenic in experimental animals and has a much longer circulating half-life and is thus much more effective as an antitumor agent.[17,19]

Toxicology testing of ADI-SS PEG 20,000 mw indicated it was safe in mice. More recently, results obtained from the testing of this drug in a single patient with HCC treated as a single patient exemption to our investigational new drug indicated that this therapy was well tolerated and produced an antitumor response.[20] Here we report the first results obtained with this drug in a larger patient population.

PATIENTS AND METHODS

Eligibility

Only patients with advanced or metastatic inoperable HCC were admitted to this study. None of these patients had any chemotherapy within 30 days before or during this study and all were older than 18 years, had a Karnofsky performance status (KPS) ≥ 40% and were treated as outpatients at the G. Pascale National Cancer Institute in Naples, Italy. This phase I/II protocol was conducted under approval of the Italian Health Ministry and the institutional review board at the G. Pascale National Cancer Institute in Naples, Italy. All patients were advised of the risks associated with their participation in this study and provided informed consent according to the Declaration of Helsinki.

Treatment Protocol

Patients were sequentially enrolled onto one of four cohorts. The first three cohorts were composed of three patients, each treated with an initial ADI-SS PEG 20,000 mw dose of 20, 40, or 80 U/m^2. Subsequent patients were enrolled onto cohort 4 and treated with an initial dose level of 160 U/m^2. This latter dose was determined to be the optimum biologic dose (OBD), defined as that amount of ADI-SS PEG that lowered plasma arginine to undetectable levels for 1 week (Table 1).[20]

Table 1. Treatment Protocol

Cohort	No. of Patients	Dose of ADI-SS PEG 20,000 mw (U/m^2)		
		Cycle 1	Cycle 2	Cycle ≥ 3
1	3	20	80	160
2	3	40	80	160
3	3	80	160	160
4	10	160	240	240

Abbreviation: ADI-SS PEG 20,000 mw, arginine deiminase conjugated to polyethylene glycol.

Because advanced HCC is uniformly fatal, it was decided that all patients should receive three cycles at the OBD, provided that the toxicity was acceptable, to assess tumor response to this therapy. Thus the dose of drug administered on second and subsequent cycles of treatment was increased as shown above. All treatments were administered by intramuscular injection. The initial cycle of treatment consisted of three treatments on days 1, 15, and 22. Subsequent cycles consisted of four treatments on days 1, 8, 15, and 22. Subsequent cycles of treatment were initiated on day 36 of the preceding cycle.

The total number of patients to be enrolled was determined as described by Simon.[21,22] This protocol design would terminate the study early if a predetermined response rate was not observed. According to this protocol design, in the first stage of the study, a predetermined number of patients were treated and if none of the patients had an antitumor response, then ADI-SS PEG 20,000 mw was to be declared inactive and the study terminated. If, however, the predetermined number of responses had been observed in the first stage of the study, then the recruitment of patients was to be continued until either the predetermined number of responses were observed (in which case ADI-SS PEG 20,000 mw was to be declared active) or the maximum number of patients allowed was reached. We chose a targeted response rate of 20%. Thus, under these statistical considerations, at least one of the first 12 patients to receive treatment at the OBD for 3 months needed to show a response or the study would be terminated and ADI-SS PEG 20,000 mw would be declared inactive. If, however, at least one of the first 12 patients responded to treatment, recruitment of patients would be continued until four patients were observed to respond (and ADI-SS PEG 20,000 mw would be declared active) or a maximum number of 37 patients had been treated for 3 months at the OBD.

Before study entry, all patients provided a complete medical history assessment of performance status and underwent physical examination, including medical laboratory studies. In addition, an abdominal computed tomography scan (CT scan) was performed on each patient within 30 days before entry onto study and repeated every 4 weeks to assess response to therapy. CT scans were performed using spiral (helical) techniques.[23-25]

Patients were examined weekly by a physician for physical examination and toxicity assessment. The National Cancer Institute Common Toxicity Criteria version 2.0 was used to describe all toxicities observed. Tumor response was determined using standard National Cancer Institute criteria, delineated as follows. Complete response was defined as disappearance of all radiologic

evidence of disease for at least 4 weeks. Partial response was defined as a greater than 50% reduction in bidimensional tumor measurements without the appearance of any new lesions for at least 4 weeks. Progressive disease was defined as either a 20% increase in the bidimensional measurements of all tumors or the appearance of any new lesion or the reappearance of any lesion that had disappeared. Stable disease was defined as tumor response that was not a complete response, partial response, or progressive disease.

Pharmacodynamics

To determine the pharmacodynamics of ADI-SS PEG 20,000 mw, amino acid analysis was performed on the plasma samples obtained at various times after administration of drug. Briefly, this is performed by collection of blood from a peripheral vein, centrifugation of the blood to obtain plasma, and then acid precipitation of the proteins from the plasma. Amino acid analysis is then performed on the resulting supernatant as previously described.[19]

Pharmacokinetics

The pharmacokinetics of ADI-SS PEG 20,000 mw were determined using two different assays as previously described.[19] The first measured the amount of ADI enzyme activity in the plasma. The second quantified the amount of ADI protein present in the plasma at each time point. This allowed us to determine whether enzymatically inactive ADI had a different circulating half-life compared with enzymatically active ADI.

Testing for Antibodies to ADI-SS PEG 20,000 mw

Two different assays were used to determine the immunogenicity of this protein in humans. The first is an enzyme-linked immunosorbent assay (ELISA), which measures the titer of antibody to ADI-SS PEG 20,000 mw. The second is an enzyme assay used to determine whether neutralizing activity is present in the plasma samples. Both assays were performed as previously described.[19]

RESULTS

Patient Characteristics

This study was performed between July 2002 and January 2003. A total of 19 patients were enrolled onto this study. The characteristics of the patients enrolled are listed in Table 2.

Table 2. Demographic Characteristics of 19 Patients With Hepatocellular Cancer Treated With ADI-SS PEG 20,000 mw

	No. of Patients	%
Total patients	19	100
Sex		
Male	17	89
Female	2	11
Age, years		
Mean	64	
Range	42-74	
Karnofsky performance status		
≥ 80%	5	26
60%-70%	10	53
40%-50%	4	21
HCC disease stage (AJCC, TNM)		
Stage III	2	11
Stage IVA	16	84
Stage IVB	1	5
Hepatitis viremia		
Hepatitis virus-free	1	5
Hepatitis virus infection	18	95
HCV, only virus	9	47
HBV + HCV	8	42
HAV, HBV, HCV	1	5
Cirrhosis (Child-Pugh classification)		
None	1	5
A	4	21
B	10	53
C	4	21

Abbreviations: ADI-SS PEG 20,000 mw, arginine deiminase conjugated to polyethylene glycol; HCC, hepatocellular carcinoma; AJCC, American Joint Committee on Cancer; TNM, tumor-node-metastasis system; HCV, hepatitis C virus; HBV, hepatitis B virus; HAV, hepatitis A virus.

Compliance

Of the 19 patients entered onto the study, 15 patients (79%) completed all cycles of treatment. Of the four patients who did not complete the study, two were discontinued from treatment because of progressive disease and two others died while on study as a result of complications of their cirrhosis (hemorrhage of esophageal varices). As noted in Table 2, 18 (94.7%) of the 19 patients in this study had biopsy-proven cirrhosis. A total of 240 treatments were scheduled, and 238 treatments (99.1%) were actually administered. The average number of treatments per patient was 12.5 (range, six to 19 treatments).

Observations Related to Safety

At no time during treatment did any of the patients complain of any severe adverse effects after treatment. The pain associated with injection was mild, and the injection site most often became tender to palpation approximately 24 hours after injection. In all instances, patients reported no tenderness by 2 to 3 days after injection.

Several clinical laboratory abnormalities were noted after ADI-PEG 20,000 mw injection and are listed in Table 3. Except for the elevation in uric acid, none of these correlated with the dose of ADI-SS PEG 20,000 mw and thus were not attributed to the treatment. The most common laboratory abnormalities observed after treatment with ADI-SS PEG 20,000 mw were increases in fibrinogen. However, in none of the patients did this increase manifest itself in coagulopathies, and this clinical observation is not graded according to the Common Toxicity Criteria.

Lipase and amylase levels were also occasionally elevated after treatment. However, there was no correlation with the dose of ADI received and the severity or incidence of this clinical laboratory abnormality. None of the patients developed clinical pancreatitis. Several patients did develop hyperuricemia after treatment. Because none of these patients had a history of gout and the hyperuricemia was always observed at ADI-SS PEG 20,000 mw dose levels ≥ 80 U/m^2, the hyperuricemia was scored as a side effect related

Table 3. Summary of Adverse Events Noted in All Patients

Adverse Event	Total No. of Toxicities Noted		
	Grade 1	Grade 2	Grade 3
Elevated serum lipase	7	0	1
Elevated serum bilirubin	3	0	0
Elevated serum creatinine	3	0	0
Elevated serum uric acid	3	0	0
Elevated serum amylase	3	0	1
Elevated serum K^+	1	0	0
Decreased serum Ca^{++}	1	0	0
Elevated serum Mg^{++}	1	0	0
Elevated serum AST	0	1	0
Decreased platelet count	1	0	0
Decreased WBC Count	1	0	0

NOTE. All toxicities were scored in accordance with the National Cancer Institute Common Toxicity Criteria Version 2.
Abbreviations: K^+, potassium; Ca^{++}, calcium; Mg^{++}, magnesium.

to treatment. Interestingly, all patients but one with elevated uric acid exhibited radiographic evidence of tumor necrosis. It was concluded that this was an adverse event associated with the antitumor effectiveness of this therapy. All patients developing hyperuricemia were promptly treated intravenously with urate oxidase (Elitek; Sanofi, Paris, France), and none developed tumor lysis syndrome.

There were no other serious adverse events observed in this study. No events were life-threatening, required inpatient hospitalization, or prolongation of existing hospitalization, and no events resulted in persistent or significant disability or incapacity.

Immunogenicity of ADI-SS PEG 20,000 mw in Patients With HCC

None of the plasma samples obtained from any of the patients inhibited the enzymatic activity of ADI-SS PEG 20,000 mw in vitro (data not shown); this result is consistent with the lack of neutralizing antibody production. Data from the ELISAs performed on all patients were combined into a single figure (Fig 1), as there was no dose effect on the titer of antibodies produced. The slight increase in anti–ADI-SS PEG 20,000 mw titers plateaued at day 20 and did not increase to higher levels in following weeks (data not shown). These results were consistent with the lack of allergic reactions observed in these 19 patients; no patient developed evidence of a systemic or local cutaneous allergic response to ADI-SS PEG 20,000 mw injections.

Pharmacodynamics

The plasma arginine concentration from each of the cohorts of patients is illustrated in Figure 2. Note that a dose of 160 U/m^2 was sufficient to eliminate all detectable arginine from the circulation for at least 7 days.

Pharmacokinetics

Pharmacokinetics of ADI-SS PEG 20,000 mw were determined using two different assays. The first assay used was

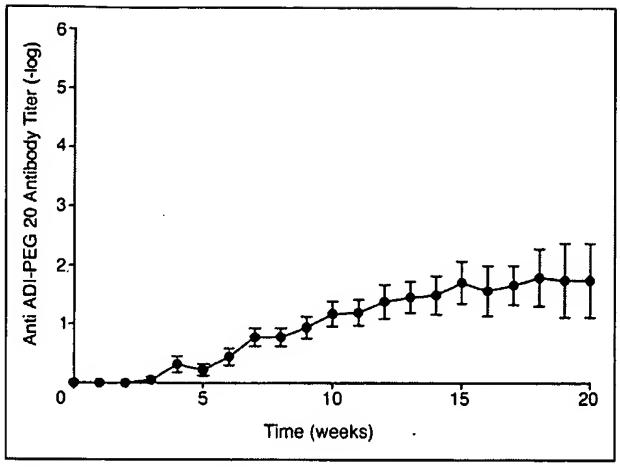

Fig 1. The immunogenicity of repeated intramuscular injections of arginine deiminase conjugated to polyethylene glycol (ADI-SS PEG 20,000 mw) in individuals with hepatocellular carcinoma. The titers of anti–ADI-SS PEG 20,000 mw antibodies have been combined into a single plot, as there were no differences in the levels of antibodies and the dose of ADI-SS PEG 20,000 mw received (data not shown).

a direct measurement of ADI enzyme activity in the plasma. The results from this assay are shown in Figure 3A. To determine whether enzymatically inactive ADI-SS PEG 20,000 mw could remain in the plasma for a longer time, a second assay used an ELISA to quantify the amount of ADI-SS PEG 20,000 mw protein present in the plasma. The results from this assay are shown in Figure 3B. Note the pharmacokinetics were similar irrespective of the assay used. When the specific activity of ADI-SS PEG 20,000 mw (12 U/mg of protein) is compared with the amount of

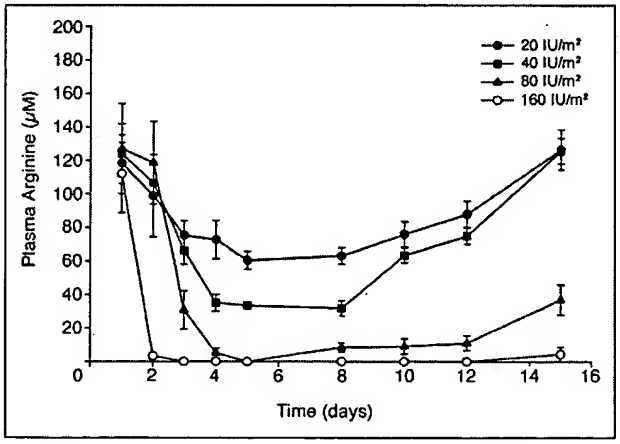

Fig 2. The pharmacodynamics of a single intramuscular injection of arginine deiminase conjugated to polyethylene glycol (ADI-SS PEG 20,000 mw) in humans with hepatocellular carcinoma. Plasma arginine levels from each of the patients at the indicated times are shown. The data shown represent the means ± SE for each of the cohorts. None of the patients treated at 160 U/m^2 of ADI-SS PEG 20,000 mw had any measurable arginine (detection limit < 2 mmol/L) in the circulation for the 7 days after treatment. Values are mean ± SEM.

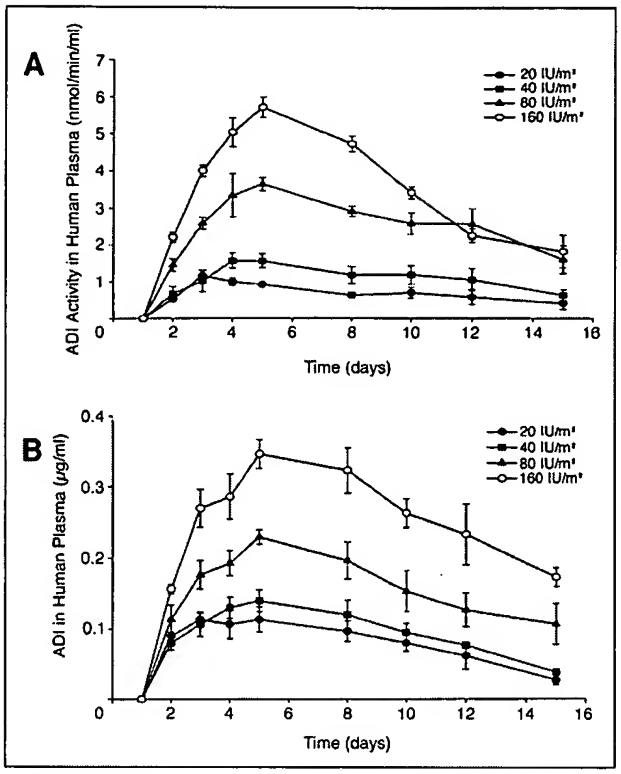

Fig 3. The pharmacokinetics of a single intramuscular injection of arginine deiminase conjugated to polyethylene glycol (ADI-SS PEG 20,000 mw) in humans with hepatocellular carcinoma. (A) ADI-SS PEG 20,000 mw pharmacokinetics determined by ADI enzyme activity in plasma. (B) Enzyme-linked immunosorbent assay performed on the same samples using an antibody to ADI-SS PEG 20,000 mw measured plasma ADI protein levels. Values are mean + SEM.

protein detected by ELISA, there is an excellent correlation between the data obtained by both of these assays.

Effects of ADI-SS PEG 20,000 mw on HCC Tumors

Results from this radiologic assessment indicated that two patients (10.5%) had a complete response, seven patients (36.8%) had a partial response, seven patients (36.8%) had stable disease, and three patients (15.9%) had progressive disease. Although complete responses to other systemic treatments for HCC have been noted in the medical literature, they are in fact quite unusual, thus the CT scans from those individuals are shown (Fig 4).

The durability of the response was also measured. Durability was defined as time from the start of treatment until progression. The mean durability of the responses noted above to the time of this report is > 400 days (range, 37 to > 680 days). Despite all treatments having been discontinued for approximately 6 months, eight patients continue to have stable disease or better, including the two patients with complete responses. The remaining six patients have developed progressive disease in the 6 months after termination of treatment.

Effects of ADI-SS PEG 20,000 mw on Performance Status and Functional Liver Reserve

The overall performance status and functional liver reserve of each patient at the time of enrollment and end of treatment was also recorded. The mean KPS at the start of the study was 66%. The mean KPS of the 19 patients at the end of treatment was 91%. The median Child-Pugh classification at the start of the study was B. The median Child-Pugh classification of the surviving patients at the end of the study was A. Note that this improvement did not seem to be a result of the patients in poorer condition dying but rather because these patients seemed to derive medical benefit from this therapy as measured by these clinical and laboratory criteria.

DISCUSSION

It has long been known that arginine is required for growth of some tumors. Gilroy[26] first demonstrated that mice fed a diet rich in arginine developed tumors that grew faster and to a larger size than mice fed a normal diet. Conversely, it was later shown that mice fed diets deficient in arginine have reduced tumor growth.[27,28] Thus there is a long history of evidence in the nutritional literature indicating a requirement for arginine in the growth of some tumors. These observations prompted several groups to use arginase as a means of decreasing arginine in both animals and in vitro.[29-31] These experiments were largely unsuccessful, as this enzyme had a weak affinity for arginine (45 mmol/L) and a nonphysiologic alkaline pH optimum (> 9.0).[32]

An independent line of evidence also implicated the essential role of arginine in tumor cell growth in that various laboratories noted the deleterious effects of *Mycoplasma* contamination on the viability of tumor cells (but not normal cells) in culture.[33] Kraemer et al[34,35] demonstrated that the growth inhibitory effects of *Mycoplasma* on tumor cells in vitro could be overcome by the addition of excess arginine to the cultures. Schimke et al[36] discovered that arginine catalysis by *Mycoplasma* occurred by a novel enzyme, ADI, which converted arginine into citrulline and ammonia and not ornithine and urea, as does arginase. It was later proven by Simbercoff et al[37] that it was ADI from the *Mycoplasma* that killed the tumor cells in contaminated cultures.

We examined a large number of ADIs purified from many different microbes and found that ADI from *M. hominus* to have the most optimal combination of physiologic pH optimum and highest affinity for arginine (approximately 10-fold greater than other *Mycoplasma* ADI enzymes). We further tested a large number of PEG formulations and found that ADI formulated with succinimydal succinimide PEG of 20,000 mw (a formulation termed ADI-SS PEG 20,000 mw) has the longest circulating half-life

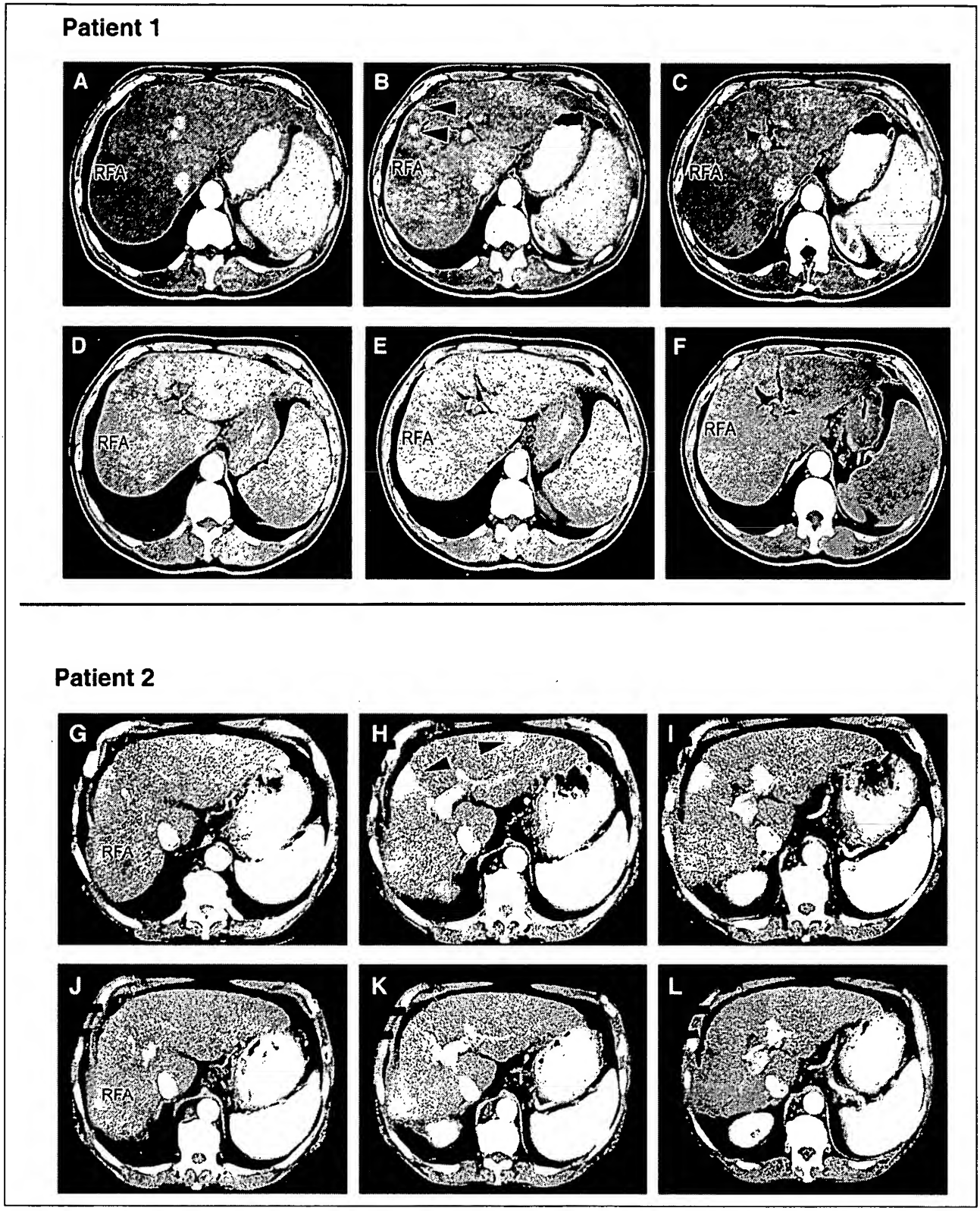

Fig 4. Computed tomography scans showing the effects of arginine deiminase conjugated to polyethylene glycol (ADI-SS PEG 20,000 mw) on hepatocellular carcinoma (arrows) in two patients with radiologic complete response. Upper three computed tomography panels for each patient, pretreatment; lower three panels, after three cycles of treatment. RFA, radiofrequency ablation.

and least immunogenicity.[19] This drug was extensively tested both in vitro and in mouse HCC xenograft models and found to have potent antitumor activity. Toxicologic testing also indicated this drug to be safe in experimental animals.

This report is the first report of the systematic testing of ADI-SS PEG 20,000 mw in human cancer patients. Analysis of safety data obtained from this study indicated that ADI-SS PEG 20,000 mw treatment was well tolerated, thus confirming the reports of Rose et al[38,39] and Snyderman et al, [40] who demonstrated that humans do not require exogenous arginine. There were no instances of ADI-SS PEG 20,000 mw treatment being discontinued or the dose lowered for safety reasons during this study. None of the patients requested cessation of treatment or removal from the protocol.

Fourteen (73.7%) of the 19 patients entered onto this study have died to date, and the median survival was 410 days. All deaths were attributed to factors other than ADI-SS PEG 20,000 mw. Two grade 3 toxicities, one grade 2 toxicity, and 24 grade 1 toxicities were observed. However, all were considered unlikely to be related to treatment and more likely related to the severity of the underlying disease, except for the three instances of elevated uric acid, which probably were related to ADI-SS PEG 20,000 mw-induced HCC tumor lysis. Testing for antibody to ADI-SS PEG 20,000 mw indicated that this drug had little immunogenicity; no neutralizing activity was found in any of the patients. These observations were consistent with the absence of any patient developing redness at the injection site, fever, rash, hypotension, shortness of breath, or other symptoms of allergic response after treatment. Our preclinical studies demonstrated that ADI-SS PEG 5000 mw was significantly more immunogenic than the 20,000 mw PEG formulation.[41] Furthermore, injection of nonpegylated ADI in our preclinical models produced a marked immune response with a greater than 10-fold increase in anti–ADI antibody titers compared with the levels seen in our patients treated with ADI-SS PEG 20,000 mw.

Treatment of patients with unresectable HCC for at least 3 months with the OBD ($\geq$ 160 U/m^2 once a week) resulted in measurable antitumor response in a cohort of the patients. Results obtained from this small study indicated a high response rate, with 16 (84.2%) of 19 patients enrolled having stable disease, partial response, or complete response. A partial response was obtained in seven of 19 patients, and two of 19 had a complete response (response rate, nine of 19 patients or 47.4%).

One of the strengths of this study is that it was conducted in patients who are representative of most patients with HCC and not a highly selected subgroup of the best patients. Thus these patients all had severe cirrhosis and advanced HCC, which is typical of most patients seen at treatment centers. In fact, more than half of these patients had Child's class B or C cirrhosis, and all but two patients had stage IV (tumor-node-metastasis system) malignant disease. Moreover, nearly half of these patients (eight of 19) had a KPS $\leq$ 60%. Previous studies of such significantly affected individuals indicate that none of these patients would have been expected to have survived more than 6 months.[8-10]

It is recognized that the data presented here are from a small patient population; nonetheless, the results are encouraging, as are those obtained to date from the ongoing phase II study currently being conducted at the University of Texas M.D. Anderson Cancer Center (Houston, TX). All these data suggest that further testing in a larger patient population is warranted. Another limitation of this study was that all patients only received three cycles of treatment at the OBD. Animal data indicate that the mechanism of this drug is the selective starvation of the tumor. Thus treatment of individuals for a longer time period may result in improved results. Recently, we have described a histochemical method for determining whether a tumor is sensitive to arginine deprivation therapy.[42] Analysis of a large number of human tumor biopsies from diverse cancers from various organs indicates that the incidence of arginine auxotrophy may be quite high, and therefore the potential may exist to use ADI-SS PEG 20,000 mw therapy in a variety of cancers in addition to HCC.

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Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Received more than $2,000 a year from a company for either of the last 2 years: C. Mark Ensor, Frederick W. Holtsberg, John S. Bomalaski, Mike A. Clark, Phoenix Pharmaceuticals.

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Utility of early testing for HCV viremia as predictive factor of sustained response during interferon or interferon plus ribavirin treatment

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Background/Aim: **To evaluate the utility of early testing for hepatitis C viremia as a predictor of treatment outcome during interferon or combination therapy.**
Methods: **We studied 184 patients with chronic hepatitis C who received interferon and were monitored for HCV RNA. Sixty-two patients received interferon alone for 12 months and 122 patients, who were still HCV RNA positive at 2 months, received an additional 12-month course of interferon and ribavirin combination therapy.**
Results: **Using this strategy, sustained response occurred in a total of 34 patients (18.5%). Independent variables associated with sustained response were HCV genotype ($p=0.06$), viral load ≤5.1 logs/ml ($p=0.005$) and negative HCV RNA at 1 month ($p<0.0001$) in the interferon group, and female sex ($p=0.04$), genotype ($p=0.03$), viral load ≤5.5 logs/ml ($p=0.01$), normal ALT ($p=0.001$) and decline in viral load ≥1.2 logs/ml after 2 months of interferon monotherapy ($p<0.001$) and negative viremia at 5 months of ribavirin onset ($p<0.0001$) in the combination therapy group. Persistence of viremia at 1 month of interferon monotherapy and at 5 months of combination therapy were the strongest predictors of non-response (negative predictive value of 100% and 99%, respectively).**
Conclusions: **Qualitative assessment of HCV RNA during treatment is the strongest predictor of sustained response during interferon or combination therapy for chronic hepatitis C.**

Key words: **HCV RNA; Interferon; Predictors of response; Response to therapy; Ribavirin.**

INTERFERON is the mainstay of treatment for chronic hepatitis C virus (HCV) infection. However, sustained response occurs in less than 30% of treated patients (1–3). The economic cost is very high, and, furthermore, it has several adverse effects. There have therefore been some attempts to choose the best candidates for interferon treatment, in order to improve cost-benefit balance. Some factors have been associated with sustained long-term response to interferon: young age, short duration of infection, absence of cirrhosis, low hepatic iron stores, low HCV RNA levels, and genotype other than 1 or 4 (1–3). Nevertheless, these predictors can hardly determine long-term outcome in an individual patient.

Several studies have investigated the utility of monitoring serum HCV RNA during interferon treatment as a predictor of sustained response (4–12). In most, early loss of detectable HCV RNA (between 1 and 4 months) is the strongest predictor of long-term sustained response. However, most of these studies have been done using home-made RT/PCR techniques that are not standardized among laboratories and require complex installations and trained personnel. Therefore, it seems difficult to generalize the use of this powerful predictor to routine clinical practice.

Recently, combination therapy with interferon and ribavirin has been shown to be more effective than interferon alone in both naive patients and those who have relapsed after interferon treatment (13–16). Although several pretreatment factors, such as HCV genotype other than 1, low base-line viral load, absence

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of cirrhosis and female sex, have been associated with sustained response, the dynamics of viral clearance during combination therapy has not been established, and the utility of monitoring for serum HCV RNA has not been assessed.

We have investigated the utility of testing for serum HCV RNA using a standardized commercially available RT/PCR test to predict therapy outcome in prospectively followed patients treated with interferon or combination therapy.

Subjects and Methods

From 1995 to 1997 we enrolled 184 chronic hepatitis C patients with indications for IFN treatment (elevated ALT values, detectable HCV RNA in serum and histological evidence of chronic hepatitis). Exclusion criteria were HIV infection, hemophilia, HBV infection, alcohol abuse, other causes of liver disease, and major contraindications to interferon therapy.

An initial observation period of 6 months was established to accomplish a clinical evaluation, monthly ALT determination, HCV genotyping and quantification of HCV baseline viremia. All patients began interferon alfa-2b (Intron-A, Schering-Plough, Kenilworth, NV, USA) treatment at standard doses (3 MU/tiw). Alanine aminotransferase (ALT) and qualitative RT/PCR for serum HCV RNA were tested every week during the first month, and biweekly during the second month of IFN therapy. At week 8, patients who had become serum HCV RNA negative were continued on IFN for another 10 months, with monthly ALT and HCV RNA determination. Those in whom HCV RNA was still detectable were offered combination treatment with IFN and ribavirin for 1 year. ALT and qualitative HCV viremia were assessed monthly.

Post-treatment follow-up period consisted of a minimum of 6 months, with monthly determination of ALT. Qualitative HCV viremia was tested 6 months after the end of therapy. A post-treatment liver biopsy was also obtained after informed consent in a subset of patients, 6 months to 1 year after completion of therapy.

In addition, quantitation of HCV viremia after the first 2 months of IFN treatment was performed in serum samples from a subset of 43 patients who had completed a year of combination treatment. In this group there was a similar proportion of sustained responders (15), relapsers (14) or non-responders (14).

HCV RNA testing

In all cases blood samples were centrifuged within 2 h of collection and the serum aliquoted and stored at −80° until further testing. Qualitative RT/PCR for HCV RNA was performed using a microwell plate-based detection test, Amplicor® HCV v1.0 (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) according to the manufacturer's instructions. In all patients the serum sample obtained at 4 weeks of treatment was also assessed with an improved second-generation RT/PCR test (Amplicor HCV v2.0). Similarly, monthly samples from patients who received combination therapy were tested with Amplicor HCV v2.0. The lower detection limit of these techniques is 10^3 copies/ml for Amplicor HCV v1.0 and less than 10^2 copies/ml for Amplicor HCV v2.0 (17).

Quantitative RT-PCR for HCV was performed using Amplicor HCV Monitor® v2.0 (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) according to the manufacturer's instructions (18). All results of quantitative HCV viremia are expressed as $\log_{10}$ copies/ml.

Viral genotype was determined with a commercial strip hybridization assay (Inno-lipa II Innogenetics, Zwijndrecht, Belgium).

Liver biopsies were examined by an expert pathologist, who gave a numeric score referring to necroinflammatory activity (Grade, from 0 to 18) and another score referring to fibrosis and architectural distortion (Stage, from 1 to 6), following the method defined by Ishaak et al. (19).

Response definitions

A patient was considered to have a sustained response when ALT values remained normal and HCV RNA undetectable for 6 months after the end of therapy. Relapse was defined as the normalization of ALT values during treatment, followed by elevation of ALT or detectable HCV RNA after stopping therapy. All other patients were considered non-responders.

Statistical analysis

Baseline data were descriptively summarized. Differences between groups were analyzed using Student's t-test and χ^2 methods, for quantitative and qualitative variables, respectively. The positive and negative predictive value, specificity, and sensitivity were assessed as previously described (20). Multivariate analysis was performed by multiple logistic regression, and stepwise discriminant-function analysis was used to predict sustained response. Differences in the histological indexes before and after therapy were analyzed by the paired t-test or the Wilcoxon rank-sum test. All statistical significance was assessed at the $p<0.05$ level. All data analyses were carried out using the SPSS for Windows, version 7.5 (Statistical Package of Services Solutions, SPSS Inc., Chicago, IL, USA).

Results

After 8 weeks of interferon treatment, HCV viremia was undetectable in 27 (15%) patients, and all of them continued interferon for 10 more months. Of the remaining 157 viremic patients, 122 were given combination treatment for a year, and 35 completed a year of interferon monotherapy (the latter did not receive RBV because they did not consent, there was a contraindication to treatment or because of discontinuity in RBV supply). Baseline characteristics of patients are summarized in Table 1.

IFN group

Of the 62 patients who received interferon alone, 13 (21%) patients achieved a sustained response, 33 (53%) became relapsers, 13 (21%) did not respond, and 3 (5%) discontinued therapy because of side-effects.

Pretreatment factors analyzed were sex, age, age at infection, duration of infection, risk factor, previous interferon therapy, baseline ALT, genotype, baseline HCV viremia, and histological indexes (Grade and Stage). Previous transfusion was associated with a lower response rate (4% *versus* 35%, $p=0.004$), whereas an HCV genotype other than 1 or 4 and a viral load of 5.1 log copies/ml or less were associated with a higher response rate (47% *versus* 12%, $p=0.006$ and 60% *versus* 14%, $p=0.005$, respectively). Other basal factors had no relation with sustained response ($p>0.05$). Multivariate analysis of pretreatment factors by stepwise logistic regression identified absence of transfusion, genotype other than 1 or 4 and viral load of 5.1 log/ml or less as independent predictors of sustained response.

Results of qualitative HCV viremia using Amplicor HCV v1.0 during IFN treatment showed that all sustained responders were serum HCV RNA negative by

TABLE 1

Baseline characteristics of patients according to treatment group*

Characteristic	Interferon (n=62)	Combination therapy (n=122)
Age (years)	39±13	46±13
Sex (males)	39 (63%)	79 (65%)
Age at infection (years)	21±12	22±14
Duration of infection (years)†	17±9	21±13
Parenteral risk factor		
Transfusion	25 (40%)	44 (36%)
Intravenous drug use	12 (19%)	10 (8%)
Unknown	23 (37%)	68 (56%)
Naive patients	52 (84%)	81 (66%)
Baseline ALT-U/ml	92±63	107±61
Genotype		
1	41 (66%)	109 (89%)
2	7 (11%)	4 (3%)
3	10 (16%)	4 (3%)
4	4 (6%)	5 (4%)
Serum HCV RNA (log 10 copies/ml)	6.0±0.8	6.3±0.7
Liver histology		
Grade‡	5.8±2	6.1±2.4
Stage§	2.4±1.3	3±1.6

* Plus-minus values are means±SD. Because of rounding, percentages may not total 100.

† The duration of infection was estimated from the date of transfusion or initial exposure to parenteral sources, and it could not be calculated for patients in whom the source of infection was unknown.

‡ Scores could range from 0 to 18, with higher scores indicating more severe necroinflammation.

§ Scores could range from 0 to 6, with higher scores indicating more severe fibrosis and architectural distortion. A value of 6 corresponds to liver cirrhosis.

week 4. Serum HCV RNA at week 4 as measured by Amplicor HCV v1.0 was a strong predictor of sustained response with a sensitivity (that is the proportion of sustained responders who had undetectable HCV viremia) of 100%, specificity (that is the proportion of relapsers and non-responders who had detectable viremia) of 74%, positive predictive value (that is the proportion of non-viremic patients who achieved a sustained response) of 52%, and negative predictive value (that is the proportion of viremic patients who did not achieve a sustained response) of 100%. When tested by the more sensitive Amplicor HCV v2.0, loss of detectable HCV RNA at 1 month was an even stronger predictor of sustained response (sensitivity of 100%, specificity of 93%, positive and negative predictive value of 81% and 100%, respectively). Fig. 1 summarizes Amplicor HCV v2.0 results at 1 month of interferon therapy. The maximum sensitivity and specificity to predict sustained response was for the association of a normal ALT value and undetectable HCV RNA by Amplicor HCV v2.0 at 4 weeks (sensitivity of 100%, specificity of 98%, positive and negative predictive value of 93% and of 100%, respectively) (Table 2).

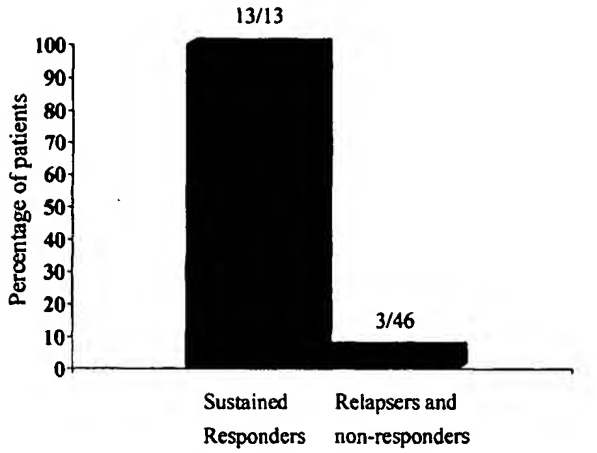

Fig. 1. Proportion of patients with undetectable serum HCV RNA by Amplicor HCV v2.0 at 1 month in the interferon monotherapy group according to treatment outcome.

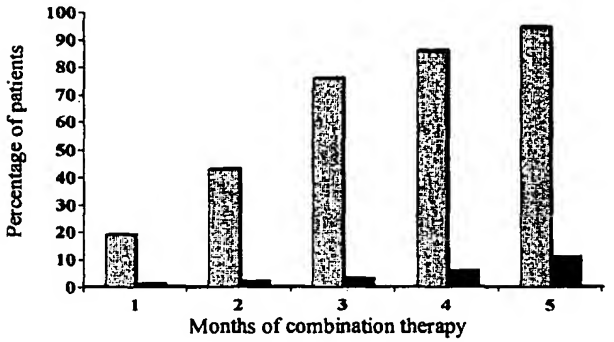

Fig. 2. Cumulative proportion of patients with a negative Amplicor HCV v2.0 test by month after onset of ribavirin in the combination therapy group according to treatment response. Grey bars summarize sustained responders' results and black bars non-sustained responders' results.

In multivariate analysis, a stepwise logistic regression identified loss of detectable HCV viremia at 1 month of treatment by Amplicor HCV v2.0 ($p<0.001$) as the stronger predictor of sustained response. Furthermore, when this variable was entered in the predictive equation, no other variable improved the prediction (Table 3).

A liver biopsy was obtained 6 to 12 months after end of therapy in 22 patients (10 sustained responders and 12 relapsers). While no significant differences on fibrosis were observed with respect to the pretreatment biopsy in any group, a significant decrease in necroinflammatory activity was observed in sustained responders (mean −4.5, 95% confidence interval (−5.6,−3.4), $p<0.001$).

TABLE 2

Utility of HCV RNA testing at 1 month of interferon monotherapy to predict sustained response. Comparison of two versions of the qualitative Amplicor HCV assay

Assay version	Sensitivity	Specificity	PPV	NPV
Amplicor v1.0	100%	72%	52%	100%
Amplicor v2.0	100%	94%	81%	100%

PPV: positive predictive value. NPV: negative predictive value.

Combination treatment group

Of the 122 patients who received IFN and RBV therapy, 21 (17%) achieved a sustained response, 66 (54%) became relapsers, 22 (18%) did not respond, and 13 (11%) discontinued treatment due to side-effects.

We evaluated pretreatment factors: sex, age, duration of infection, age of infection, risk factor, previous interferon therapy, baseline ALT, genotype, baseline HCV viremia, and histological indexes (grade and stage) as predictors of sustained response. In univariate analysis, an HCV genotype other than 1 or 4 (57% *versus* 17%, $p=0.035$), a baseline viral load of 5.5 log per ml or less (44% *versus* 14%, $p=0.007$), female sex (29% *versus* 13%, $p=0.04$), and a histological grade higher than 6 (31% *versus* 12%, $p=0.03$) were associated with a higher likelihood of sustained response. Other basal factors had no relation with sustained response ($p>0.05$). Logistic regression analysis of baseline variables identified: genotype other than 1 or 4, a baseline viral load of 5.5 log per ml or less, and female sex as independent predictors of sustained response.

In addition to baseline factors, three additional variables obtained at 2 months of IFN monotherapy were also associated with a higher sustained response rate after combination therapy: normalization of ALT level (42% *versus* 14%, $p=0.02$), a viral load of 5.1 log/ml or less (70% *versus* 13%, $p<0.001$) and a decline in viral load with respect to pretreatment values of 1.2 log/ml or more (80% *versus* 12%, $p<0.001$). When these variables were entered in a multivariate analysis along with pretreatment variables, a decline in viral load of 1.2 log/ml or more ($p<0.001$) was the strongest predictor of a sustained response, followed by normalization of ALT level ($p=0.001$).

Fig. 2 shows the dynamics of viral clearance during combination treatment, in patients who had a sustained response and those who had a relapse or non-response. All but one of the sustained responders had undetectable HCV RNA 5 months after ribavirin onset.

Serum HVC RNA as measured by Amplicor HCV v2.0 at 5 months of combination therapy was a strong predictor of sustained response ($p<0.001$), with a sensi-

TABLE 3

Distribution of predictors in the group of IFN monotherapy according to response to treatment*

Characteristic	Sustained response ($n=13$)	Relapse or non-response ($n=46$)	p
Age (years)	36±13	39±12	0.35
Sex			
Males	7 (19%)	30 (81%)	0.5
Females	6 (27%)	16 (73%)	0.5
Age at infection (years)	19±3	20±14	0.8
Duration of infection (years)	14±7	18±9	0.3
Parenteral risk factor:			
Transfusion	1 (4%)	24 (96%)	0.004
Intravenous drug use	4 (33%)	8 (67%)	0.004
Unknown	8 (36%)	14 (64%)	0.004
Naive	12 (24.5%)	37 (75.5%)	0.5
Treated	1 (10%)	9 (90%)	0.5
ALT (U/ml)	94±63	88±64	0.7
Genotype:			
1 or 4	5 (12%)	37 (88%)	0.006
2 or 3	8 (47%)	9 (53%)	0.006
Serum HCV RNA (log 10 copies/ml)			
≤5.1	6 (60%)	4 (40%)	0.005
>5.1	7 (14%)	42 (86%)	0.005
Grade	6.2±2	5.9±2	0.8
Stage	2.5±1	2.5±1	0.9
HCV RNA at week 4 measured by Amplicor HCV v2.0			
Negative	13 (81%)	3 (19%)	<0.001
Positive	0	43 (100%)	<0.001

* Plus-minus values are means±SD. Percentages refer to the characteristic that defines the row.

TABLE 4

Predictive values for sustained response of qualitative HCV RNA testing at months 1 to 5 after ribavirin onset. Comparison of two versions of the Amplicor HCV test

	Month	1	2	3	4	5
Amplicor v1.0	PPV	67%	50%	53%	44%	38%
	NPV	85%	88%	95%	96%	98%
Amplicor v2.0	PPV	80%	82%	84%	78%	67%
	NPV	85%	90%	94%	96%	99%

PPV: positive predictive value. NPV: negative predictive value.

tivity of 95%, specificity of 89%, positive predictive value of 67% and negative predictive value of 99%. The corresponding figures for a negative Amplicor HCV v1.0 test result at five months of combination therapy were 95%, 64%, 38%, and 98%, respectively (Table 4).

A stepwise logistic regression including all the significant variables identified qualitative HCV viremia at the fifth month of combination therapy ($p<0.001$) as the most powerful predictor of sustained response, followed by a decline in viral load of 1.2 log/ml or more after 2 IFN months ($p<0.001$). After these two variables were entered in the logistic equation, the addition of other variables could not improve the prediction (Table 5).

Fifty-six post-treatment liver biopsies were available in the combination therapy group, 13 from sustained responders, 38 from relapsers and 5 from non-responders. There was no difference in relation to stage pre- and post-treatment in any group. Grade improved significantly in sustained responders after therapy (mean −3.6, 95% confidence interval (−4.6,−2.7), $p<0.001$), while there were no differences in relapsers and non-responders.

Discussion

In order to improve the efficacy of antiviral therapy for chronic hepatitis C, several studies have investigated pretreatment factors associated with a higher likelihood of response (21–24). In our series two pretreatment factors: low baseline viral load and HCV genotype other than 1 or 4 were associated with a higher sustained response rate, both in patients treated with

TABLE 5

Distribution of predictors in the group of combination therapy according to response to treatment*

Characteristic	Sustained response (n=21)	Relapse or non-response (n=88)	p
Age (years)	47±12	44±12	0.4
Sex			
Males	9 (13%)	59 (87%)	0.04
Females	12 (29%)	29 (71%)	0.04
Age at infection (years)	23±9	20±16	0.6
Duration of infection (years)	23±12	22±13	0.9
Parenteral risk factor			
Transfusion	8 (19%)	34 (81%)	0.5
Intravenous drug use	1 (11%)	8 (89%)	0.5
Unknown	12 (21%)	46 (79%)	0.5
Naive	16 (22%)	58 (78%)	0.5
Treated	5 (14%)	30 (86%)	0.5
ALT-U/ml	99±69	100±58	0.9
Genotype			
1 or 4	17 (16%)	85 (84%)	0.035
2 or 3	4 (57%)	3 (43%)	0.035
Serum HCV RNA (log 10 copies/ml)			
≤5.5	8 (44%)	10 (56%)	0.007
>5.5	13 (14%)	78 (86%)	0.007
Grade			
>6	15 (32%)	32 (68%)	0.03
<6	8 (13%)	53 (87%)	0.03
Stage	3.1±1.5	2.9±1.5	0.6
ALT after IFN×2m			
Normal	13 (42%)	18 (58%)	0.02
Elevated	11 (14%)	67 (86%)	0.02
HCV RNA decline after IFN×2m-log10 copies/ml			
≥1.2	12 (80%)	3 (20%)	<0.001
<1.2	3 (12%)	25 (88%)	<0.001
HCV RNA at fifth month by Amplicor HCV v2.0			
Negative	20 (65%)	11 (35%)	<0.001
Positive	1 (1%)	77 (99%)	<0.001

* Plus-minus values are means±SD. Percentages refer to the characteristic that defines the row.

interferon alone and in patients treated with interferon and ribavirin. However, as in other published studies, the prognostic value of these factors is of limited value in individual cases (25).

In contrast, our study confirms previous observations that qualitative HCV viremia at 1 month of interferon therapy is a very strong predictor of treatment outcome. Using a standardized commercially available RT/PCR test with a lower limit of detection of less than 100 copies per ml, all sustained responders had undetectable viremia by 4 weeks of interferon treatment. The fact that none of the patients who were HCV RNA positive at 8 weeks and continued interferon monotherapy for 12 months became sustained responders, further supports the utility of early monitoring for HCV viremia. The predictive value of viremia could be improved when combined with ALT value, so that 93% of the patients who had normal ALT level and undetectable HCV RNA at 4 weeks became sustained responders.

In the present study the predictive value for sustained response of a negative Amplicor v2.0 test result at 1 month, was in fact higher than that reported for other qualitative RT/PCR assays and similar to that reported for a quantitative decline in HCV RNA level of 3 or more logs/ml at 4 weeks of interferon monotherapy (26).

Based on these results, management of HCV-infected patients following interferon monotherapy could be improved by monitoring HCV RNA at 1 month by Amplicor v2.0. For those with a negative result, a complete 12-month course of interferon monotherapy would clear infection in more than 80% of patients. For patients with a positive test result at 1 month, addition of ribavirin should be considered.

Because of the study design we were able to analyze early predictive factors of eventual response in those patients who were given additional therapy with ribavirin after 2 months of interferon monotherapy. Although by definition none of these patients cleared HCV viremia during the first 2 months, those who had a decline in viral load of 1.2 log/ml or more during initial therapy were more likely to become sustained responders after addition of ribavirin. The likelihood was even higher for those who had also normalized ALT levels. These data suggest that, in fact, response to interferon plus ribavirin therapy could be better predicted on the basis of early response to interferon treatment. This finding would be in agreement with the observation that relapsers after interferon monotherapy are more likely to respond to combination treatment than naive patients.

During combination treatment, viremia was not as early a predictor of sustained response as in interferon monotherapy. Sustained responders in the combination therapy group took an average of 2.9 months to clear viremia after initiation of ribavirin. The reason for this slower clearance is probably related to different modes of action of interferon and ribavirin. These results, however, cannot be generalized to all patients on combination therapy, since our patients were given ribavirin precisely because they had failed to clear HCV RNA after 2 months of interferon monotherapy. Hence, the precise timing at which persistence of a positive Amplicor v2.0 test may have the strongest negative predictive value might be different in patients given combination treatment from the start. Nonetheless, it seems evident that, irrespective of treatment schedule, sustained responders after combination therapy may take longer to clear viremia than those treated with interferon monotherapy. Additional studies should be carried out to establish the most cost-effective strategy to monitor patients on standard combination therapy.

In addition it must be remembered that most (93%) of our patients on combination therapy were infected with HCV genotypes 1 or 4, and the dynamics of viral clearance among patients infected with other genotypes might be different.

In summary we have shown that monitoring for serum HCV RNA with a readily-available and simple-to-use qualitative RT/PCR test may be useful to tailor antiviral treatment in patients with chronic hepatitis C.

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REVIEW ARTICLE

Argininosuccinate synthetase from the urea cycle to the citrulline–NO cycle

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Argininosuccinate synthetase (ASS, EC 6.3.4.5) catalyses the condensation of citrulline and aspartate to form argininosuccinate, the immediate precursor of arginine. First identified in the liver as the limiting enzyme of the urea cycle, ASS is now recognized as a ubiquitous enzyme in mammalian tissues. Indeed, discovery of the citrulline–NO cycle has increased interest in this enzyme that was found to represent a potential limiting step in NO synthesis. Depending on arginine utilization, location and regulation of ASS are quite different. In the liver, where arginine is hydrolyzed to form urea and ornithine, the *ASS* gene is highly expressed, and hormones and nutrients constitute the major regulating factors: (a) glucocorticoids, glucagon and insulin, particularly, control the expression of this gene both during development and adult life; (b) dietary protein intake stimulates *ASS* gene expression, with a particular efficiency of specific amino acids like glutamine. In contrast, in NO-producing cells, where arginine is the direct substrate in the NO synthesis, *ASS* gene is expressed at a low level and in this way, proinflammatory signals constitute the main factors of regulation of the gene expression. In most cases, regulation of *ASS* gene expression is exerted at a transcriptional level, but molecular mechanisms are still poorly understood.

Keywords: argininosuccinate synthetase; urea cycle; arginine; citrulline-NO cycle; transcription regulation; DNA binding sequences.

Argininosuccinate synthetase (ASS, L-citrulline, L-aspartate ligase, EC 6.3.4.5) was first identified 50 years ago in the liver [1] but was more recently recognized as a ubiquitous enzyme in mammals. The enzyme catalyses the reversible ATP-dependent condensation of citrulline with aspartate to form argininosuccinate in an ordered reaction as shown below:

$$MgATP^{2-} + \text{citrulline} + \text{aspartate} \Longleftrightarrow \text{argininosuccinate} + MgPP_i + AMP$$

Argininosuccinate is the immediate precursor of arginine leading to the production of urea in the liver and that of NO in many other cells. The importance of both the hepatic and ubiquitous enzyme is, respectively, underlined by ASS deficiency, a rare genetic disorder associated with high mortality, resulting in citrullinemia in human [2,3] and by ASS over-expression leading to an enhanced capacity for NO production [4,5]. Concerning urea synthesis, the reaction catalysed by ASS is a well-known regulatory step and has therefore been studied extensively. By contrast and concerning NO production, research focused initially on NO synthase and its different isoforms but not on ASS. However, a renewal of interest in the regulation of ASS recently appeared resulting from the report of a rate-limiting role of ASS for high output NO synthesis [4]. Finally, the regulation of extra-hepatic ASS appears quite different from that reported for the liver enzyme and, concerning NO production, a coregulation of ASS and NO synthase by immunostimulants has been reported in various cultured cells and tissues.

The aim of this review is to summarize the knowledge acquired on cell/tissue specific regulation of ASS, firstly, in regards to its physiological role and, secondly, at the gene level. For recent system-focused reviews, the reader may refer to the reviews of Wu & Morris, 1998 [6], Wiesinger, 2001 [7] and Morris, 2002 [8] for arginine metabolism and that of Takiguchi & Mori,1995 [9] for the urea cycle.

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Abbreviations: ASS, argininosuccinate synthetase; NOS, nitric oxide synthase; Octn2, organic cation carnitine transporter; AP-1, activator protein 1; LPS, lipopolysaccharide; Sp 1, specificity protein 1; C/EBP, CCAAT/enhancer binding protein; HNF1, hepatocyte nuclear factor 1; ATF, activating transcription factor; AARE, amino acid response element; CTLN1, type 1 citrullinemia.

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The ASS protein

ASS, a ubiquitous enzyme

It was established many years ago that ASS activity was present in many tissues with the highest values found in the liver and kidneys [10,11], and this was confirmed recently at both mRNA and protein levels [12]. Concerning such a

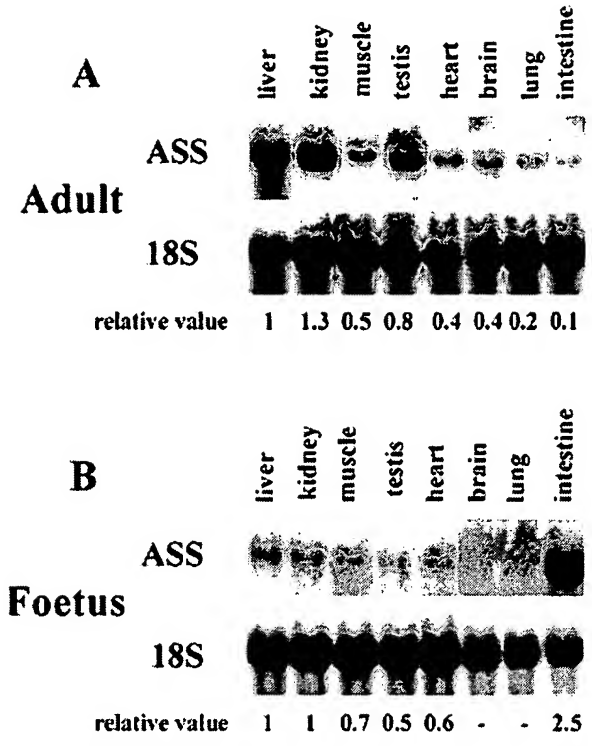

Fig. 1. Tissue distribution of *ASS* mRNA during adult and fetal periods in rats. Total RNA (25 μg per lane) was prepared from various tissues of adult (A) and 19.5-day-old fetuses (B) rats, and analysed by Northern blot (see [110] for experimental protocol). Hybridizations were performed successively with the *ASS* cDNA and the 18S rRNA probe as an internal standard. Scanned values are expressed relative to that of liver.

repartition and as illustrated in Fig. 1A for adult rat, we observed that *ASS* mRNA is expressed in all the tissues tested but with a very low expression in intestine. By contrast, the highest value was observed in intestine in rat foetus (Fig. 1B). The physiological significance of such a change in *ASS* gene expression during development is described below. More recently, it was established that the *ASS* gene is expressed in a number of cells including bovine aortic endothelial cells [13]; mouse [14] and rat macrophages [15]; rat and human pancreatic cells [16,17]; rat vascular smooth muscle cells [18] and various cell lines [19–21]. Finally, *ASS* was also detected recently in rat eye cells [22] and in glial cells and neurones (reviewed in [7]). All together, these results lead to the notion that ASS is a ubiquitous enzyme.

Within tissues however, ASS appears differently localized. For example, ASS is clearly a cortical enzyme in the rodent kidney [23,24]; in the rat liver, the enzyme appears mainly in periportal hepatocytes, according to their specific role in urea production, declining toward perivenous hepatocytes [25,26]. Such a zonation was also reported in the developing rat intestine where ASS is located mainly in the upper part of the villi, declining toward the intervillus region [27]. However, this may be, at least in part, species-dependent as such a marked zonation was not reported in human liver [28].

ASS, a highly conserved enzyme

Firstly purified from porcine kidney [29] and bovine liver [30], the enzyme was then purified to homogeneity not only from rat [31] and human liver [32], but also from human lymphoblast [33], from yeast [34] and very recently from bacteria [35]. ASS is a homotetramer, each subunit being composed of 412 amino acid residues [36] with a high sequence identity between human [37], bovine [38], rat [39] and mouse [40], as shown by the comparison of the cDNA sequences.

The kinetic properties of ASS have been studied extensively and are out the scope of this review (reviewed in [3,10,41]). It should however, be pointed out that the reaction proceeds by ordered binding and release of substrates and products as indicated in the introduction section. Although the rat liver enzyme was shown to exhibit negative cooperativity for each substrate [42], this phenomenon was controversial for the bovine enzyme [43,44] and not observed in the human [32,42]. Such a phenomenon has never been linked to the intracellular regulation of ASS activity. Interestingly, the crystal structure of the bacterial enzyme has been established recently [45], the ordered mechanism confirmed and the conformational changes described [46]. Finally, except for the report of an *in vitro* activation of ASS by thioredoxins purified from rat liver [47], no other post-translational modifications of the protein have been described. This therefore underlines the importance of the regulation of ASS at a pretranslational level.

ASS, a targeted protein

Initially described as a cytosolic liver enzyme [10,11], subcellular fractionation studies revealed that a part of the enzyme was linked to the outer membrane of mitochondria [48], and this was associated with a similar location of the *ASS* mRNA [49]. Moreover, such an intracellular repartition changes during development: indeed, 90% of the enzyme is linked to mitochondria in fetal liver but only about 30% in adult liver [48]. Such a repartition therefore contributes to the channelling of urea cycle intermediates in adult liver [50,51]. Although hormones were responsible for the change in the liver *ASS* expression (see above), the molecular mechanism leading to changes in intracellular location of the enzyme is not known.

Similarly, in ASS-transfected endothelial cells, the enzyme shows a predominant mitochondrial membrane association [4]. However it was reported recently that ASS is localized close to the plasma membrane in bovine aortic endothelial cells, a NO-producing cell [52]. Moreover in neurones, ASS appeared localized mainly in axoplasma [53]. In other cells, such as enterocytes [54] or kidney proximal convoluted tubule cells [23], ASS is clearly a cytosolic enzyme. Taken together, these results therefore suggest that the intracellular ASS location may depend on its physiological function (see next paragraph).

Cell/tissue specific regulation

ASS activity which leads to arginine synthesis contributes to three major different functions in the adult organism depending on the cell/tissue considered, as illustrated in

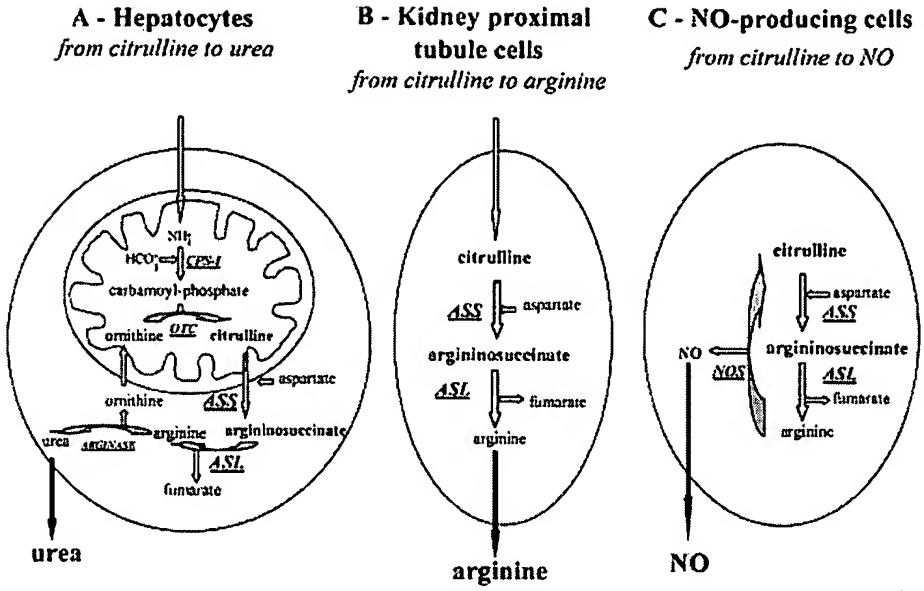

Fig. 2. Schematic representation of the three major functions of ASS in the mammalian organism. Enzymes are: CPS-I, carbamoyl phosphate synthetase-I (EC 6.3.4.16); OTC, ornithine transcarbamylase (EC 2.1.3.3); ASS, argininosuccinate synthetase (EC 6.3.4.5); ASL, argininosuccinate lyase (EC 4.3.2.1); NOS, nitric oxide synthase (EC 1.14.13.39).

Fig. 2 [(A) ammonia detoxification in the liver (B) arginine production for the whole organism by kidney cortex and (C) arginine synthesis for NO production in many other cells]. Beside these three major functions, it was suggested that ASS plays a role in neuromodulation through the production of argininosuccinate, that is a putative neuromodulator [55]. The regulation of ASS in the liver appears quite different from that reported in other cells or tissues, and we firstly describe the regulation of ASS as a key step in urea production. Secondly, we describe the regulation of ASS as a key step in arginine production for the whole organism (i.e., by the small intestine in developing animal and by the kidney in adult). Finally, we describe the regulation of ASS as a potential limiting step in NO production.

ASS, a key step in urea production

As with numerous liver genes, the *ASS* gene expression is subject to both hormonal and nutritional regulation.

Concerning hormonal regulation, a major contribution comes from studies on rodents and concerns the transition from the fetal to the postnatal animal that is characterized by an increase in the plasmatic concentration of both glucocorticoids and glucagon, and by a decrease in that of insulin [56,57]. This approach in rodents firmly established that (a) the *ASS* gene is expressed a few days before birth and (b) the developmental increase in ASS activity paralleled that of the mRNA level [58–60]: *ASS* gene expression increases progressively towards birth reaching about 50% of the adult value, as illustrated in Fig. 3 for rat liver. Such a profile in the expression of *ASS* during development was also reported in the human fetal liver where ASS activity was measurable as soon as the ninth week of gestation [61], increasing progressively and reaching 53% of the adult value at the thirteenth week of gestation and 90% at the thirty-sixth week [62]. Thus, first studies focused on the potential stimulating role of glucocorticoids, showing an increase in ASS activity by using *in vivo* approaches (i.e., newborn adrenalectomy [63], fetal hypophysectomy [64] or *in utero* injection of glucocorticoids [65]) and *in vitro* approaches (i.e., fetal liver explants [58,66] and cultured fetal hepatocytes [67]). Such a stimulating effect of glucocorticoids was also reported in adult rat liver [68,69] and cultured hepatoma cells [70], although only a slight or no effect was reported in perifused [71] and cultured adult rat hepatocytes

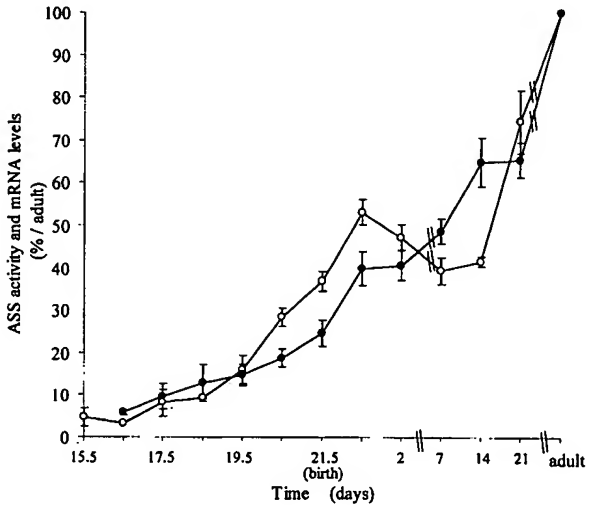

Fig. 3. Change in *ASS* expression during development in the rat liver. Levels of mRNA (open circles) and enzyme activity (black circles) are shown. Data are from [60,110]. Adult values were taken as reference (100%); ASS activity in adult was 110.9 ± 11.7 $U \cdot g^{-1}$ liver, $n = 7$.

[72,73], respectively. The effect of glucocorticoids on ASS activity was associated with an increase in the mRNA level [73–75] resulting from an increased *ASS* gene transcription [60]. However, the molecular mechanism at the gene level is not yet determined (see ASS, an unsual promoter, below). In this context, it is interesting to note that the response to glucocorticoids was inhibited partially by cycloheximide, an inhibitor of protein synthesis, suggesting the involvement of a new synthesized protein factor for full ASS induction [60,71,73,75].

Such an approach also established that pancreatic hormones, namely insulin and glucagon, play a key role in the developmental regulation of *ASS* gene expression by modulating the glucocorticoids effect. Indeed, *in utero* studies showed that (a) cortisol and glucagon act synergistically to increase ASS activity [65] and (b) insulin counteracts the effect of cortisol [76]. Finally, *in vitro* studies confirmed such an effect of pancreatic hormones during development [77,78] and we specified that the hormones act at the mRNA level [60], as illustrated in Fig. 4. In adult liver, glucagon alone increases ASS activity [79,80] possibly through an increase in cAMP: indeed cAMP analogs enhanced ASS mRNA levels both *in vivo* [74] and *in vitro* [75] by acting at a transcriptional level [74]. However, as for glucocorticoids, the molecular mechanism at the gene level remains to be established (see ASS, an unusual promoter, below). Concerning insulin action, no clear effect on *ASS* gene expression in normal adult rat was reported. However, ASS activity was increased in diabetes [79] and we recently observed, by using streptozotocin-treated rats, that insulin administration restored both ASS mRNA and activity at a physiological level (A. Husson, unpublished data). Again, the molecular mechanism at the gene level remains to be established. Finally, growth hormone was reported to decrease ASS activity and mRNA level [68,81] and could counteract the stimulating effect of prednisolone [81], contributing therefore to its reducing effect on the conversion of the amino N to urea [82,83].

Thus, stimulating hormones, glucocorticoids and glucagon, and an inhibiting hormone, insulin, are important factors for the induction of the late fetal liver enzyme, further acting on the liver enzyme throughout the adult life. Moreover, the inhibitory effect of growth hormone on *ASS* gene expression might constitute a novel mechanism of its well known anabolic action [82].

Concerning nutritional regulation, it is well established that nutritional status (protein intake or starvation) modulates ASS activity [84–86]. Although both enzyme synthesis and degradation were shown to be involved in this phenomenon [87], no data on ASS degradation is available in the literature except for some differing results on the half-life of the rat enzyme [88,89]. Protein intake was reported to increase both ASS activity and amount [89], and this was correlated to an increase in the mRNA level [74]. An *in vivo* study, particularly, demonstrated that some amino acids were effective to increase ASS activity such as alanine, glycine, glutamine and methionine in a decreasing order of efficiency [90] but the mechanism involved could not be separated from the hormonal effects. Glutamine, however, was shown to increase both ASS activity and mRNA level in cultured hepatocytes from fetal and adult rats [91]. Concerning the molecular

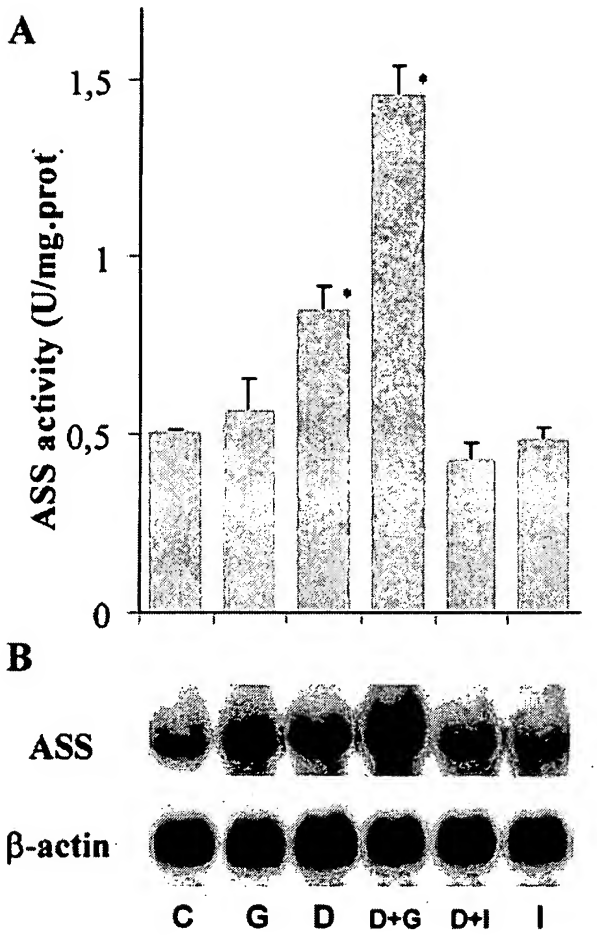

Fig. 4. Influence of glucocorticoids and pancreatic hormones on ASS expression in cultured 18.5-day-old rat hepatocytes. (A) ASS activity, means ± SEM. *Significantly different from control cells $P < 0.05$. (B) *ASS* mRNA level. Representative autoradiogram (25 μg total RNA per lane). C, control cells; D, dexamethasone 10^{-6} M; G, glucagon 10^{-7} M; D + G, dexamethasone + glucagon; D + I, dexamethasone + insulin; I, insulin 10^{-7} M. Data are from [76,78] and [60].

mechanism involved, such a stimulatory effect was, at least in part, due to the cell swelling induced by the sodium-dependent cotransport of the amino acid [91] potentially acting at a transcriptional level [92]. Interestingly, we also observed recently such a stimulatory effect of glutamine by using Caco-2 cells, a human intestinal cell line. But, in this case, this was apparently not linked to cell swelling, as shown in Fig. 5. This suggests that glutamine may regulate gene expression through different mechanisms depending on the model used (i.e., normal cells or cell lines), as proposed previously for its effect on the phospho*enol*pyruvate carboxykinase (*PEPCK*) gene regulation [93]. However, the molecular mechanism of glutamine action at the gene level is not determined. Beside amino acids, oleic acid was shown to inhibit the induction of the *ASS* gene by glucocorticoids in cultured hepatocytes [94]. Such a role of fatty acids was also underlined by studies on juvenile visceral steatosis (JVS)

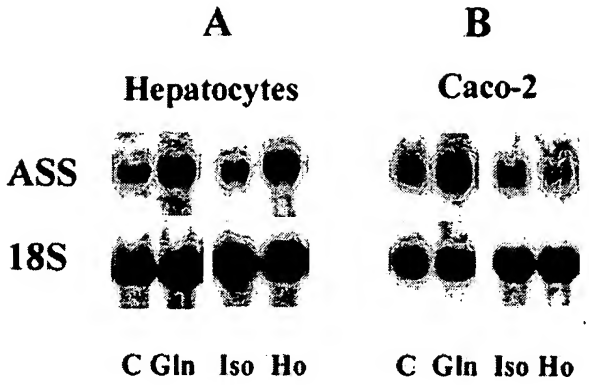

Fig. 5. Comparison of the effect of glutamine and hypoosmolarity on *ASS* expression in fetal rat hepatocytes and Caco-2 cells. Hepatocytes from 18.5-day-old fetuses and Caco-2 cells, a human enterocyte cell line, were cultured for 24 h in iso-osmotic medium with (Gln) or without (C) 10 mM glutamine and in iso-osmotic (Iso) or hypo-osmotic (Ho) medium obtained by decreasing by 50 mM the NaCl concentration. Total RNAs were extracted from cells and subjected to Northern analysis (25 μg per lane). Samples were hybridized successively with a probe for the *ASS* cDNA and for the 18S rRNA as internal standard. Representative autoradiograms are shown. (A) Hepatocytes, data are from [91]. (B) Caco-2 cells (American Tissue Culture Collection, Rockville, MD, USA) were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) without fetal bovine serum, after 2 days of confluence, between passages 30–60. Scanned values are: C or Iso, 100%; Gln, 172 ± 21%* ($n = 6$); Ho, 63 ± 7%* ($n = 4$); *statistically significant vs. C or Iso ($P < 0.05$).

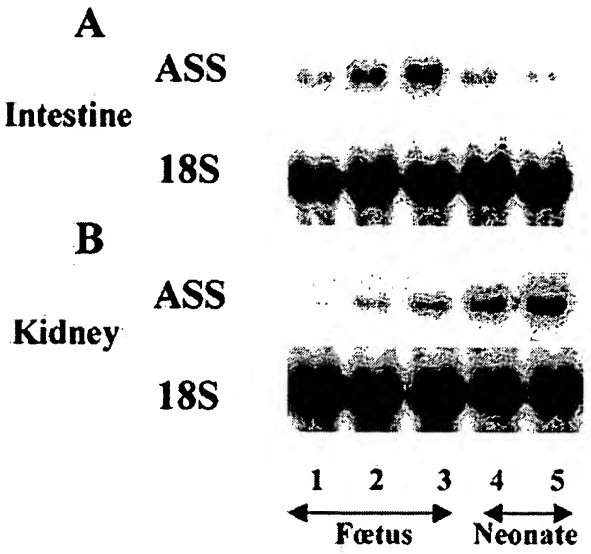

Fig. 6. Perinatal evolution of *ASS* expression in rat intestine and kidney. Total RNAs from fetal and newborn rats were extracted from ileum and total kidney, and analysed by Northern blot (25 μg per lane). Samples were probed successively with the *ASS* cDNA and the 18S rRNA as internal standard. Representative autoradiogram: Lane 1, 17.5-; lane 2, 19.5-; lane 3, 21.5-day-old fetuses; lanes 4 and 5, 3 week- and 5 week-old neonates, respectively.

mice that are deficient in carnitine due to a defect in the *Octn2* gene encoding a high affinity carnitine transporter [95]. They present an alteration in the urea cycle enzymes including ASS [96] and the expression of the *ASS* gene, for example, was restored in mutated mice receiving carnitine [97]. In these mice, and concerning another key enzyme of ureagenesis, namely carbamoylphosphate synthetase (CPS), it was demonstrated recently that fatty acids act through an interaction between glucocorticoids and AP-1 [98]. However, this remains to be confirmed for ASS. For further details on the regulation of the five urea cycle enzymes, see [8,9,99,100].

Thus, nutrients such as glutamine or fatty acids are able to regulate the expression of the hepatic *ASS* gene, but the molecular mechanism involved is not clearly established.

ASS, a key step in arginine production

Arginine is not only recognized as an essential amino acid in foetuses and neonates, but also as a conditionally essential amino acid in adults, particularly in some pathological conditions [6,101,102]. Although numerous cell/tissues are able to synthesize arginine, it is well established that small intestine is the major site of its synthesis during the developmental period and shifts to citrulline production thereafter, in rodents as in humans [103–105]. Initially expressed in enterocytes during the developmental period, intestinal ASS progressively disappeared but appeared in the kidney [106–108], establishing an 'intestinal–renal' arginine biosynthetic axis in adult [6,102], as illustrated in Fig. 6 for the rat ASS mRNA. In developing kidney, the appearance of the enzyme activity is directly linked to that of the mRNA [24,109,110] through an activation of transcription of the *ASS* gene, as seen in the liver [110]. In contrast to liver however, the factors modulating *ASS* gene expression are not known both in enterocytes and kidney cells. Indeed, glucocorticoids neither affected ASS activity in porcine enterocytes [111] nor modulated the *ASS* mRNA level in kidneys of both newborn [110] and adult rats [112]. Finally, protein deprivation did not change renal ASS activity [113], although an increase in mRNA level was reported [112]. All the obtained results clearly demonstrate that the regulation of ASS in intestine and kidney is different from that reported in the liver. This was also confirmed in mice homozygous for deletions overlapping the albino locus on chromosome 7 [114]. Indeed, in these mice, transcription of the *ASS* gene and mRNA level were reduced in the liver, but not in kidney [114].

Although the importance of both intestinal and renal ASS has been recognized for a long-time, factors including hormones and nutrients have not yet been identified as inducers of the gene expression.

ASS, a potential limiting step in NO production

Beside its hydrolysis catalysed by arginase (EC 3.5.3.1) leading to ornithine and urea production, arginine is a substrate of NO synthase (NOS, arginine deiminase, EC 1.14.13.39) leading to citrulline and NO (see Fig. 2A and C, respectively). Citrulline, through the reactions

catalysed by ASS and argininosuccinate lyase (ASL, EC 4.3.2.1) may cycle back to arginine, constituting an arginine–citrulline cycle [18,115] also called the citrulline–NO cycle (Fig. 2) [6,102]. Three isoforms of NOS catalyse the reaction: the endothelial constitutive NOS (eNOS), the neuronal constitutive NOS (nNOS) and the inducible NOS (iNOS), reviewed in [116,117], but research mainly focuses on iNOS as the expression of this isoform is induced by proinflammatory stimuli. Then, coinduction of iNOS and ASS was demonstrated *in vivo* in various tissues including heart, kidney, lung and spleen by using LPS-treated rats [118,119]. Such a coinduction was also obtained in various LPS- and/or cytokine-stimulated cells in culture [14,17,18] including different cell lines [20,21,120] and different kind of cells of the nervous system [121–123]. In neurones and glial cells of rodent and human brains [121–126], both *iNOS* and *ASS* were shown to be increased by LPS and/or cytokines, but some cells in the nervous system did not express both enzymes, suggesting the existence of an intercellular citrulline–NO cycle [7,126]. This point, however, remains to be firmly established. Finally, the importance of ASS in NO-producing cells was confirmed in transfected cells: in iNOS-transduced endothelial cells, an enhanced ASS activity has been reported resulting in a sustained NO production even in nonstimulated cells [127]. Moreover, in ASS-transfected smooth muscle cells, an increased capacity for immunostimulant-induced NO synthesis was observed [4]. Thus, LPS and various proinflammatory cytokines, including IL-1β, IFN-γ or TNF-α, increase ASS both at mRNA and protein levels, and a transcriptional effect was suggested [17,18]. Moreover, such a stimulating effect of LPS and cytokines on the ASS mRNA level was inhibited by the addition of glucocorticoids in vascular smooth muscle cells and endothelial cells [18,128].

Other regulatory factors, such as amino acids, were shown to inhibit the *ASS* gene expression in other cells. Indeed, glutamine as arginine decreases ASS activity in cultured endothelial cells [13,129,130], and in human and mouse cell lines [131]. Concerning arginine, de-repression of *ASS* mRNA level and activity was reported by culturing human lymphoblasts and RPMI-2650 cell line in the absence of the amino acid or by using canavanine resistant cells [132–134], and this involved an increase in gene transcription [135]. However, the link between ASS and iNOS has not been thereafter studied. Additionally, NH_4Cl was reported to stimulate ASS in cultured rat astrocytes [136] and some other regulatory factors, such as TGF-β [137] and shear stress [138] were recently shown to stimulate *ASS* gene expression in rat and human cultured endothelial cells, respectively.

In conclusion, various factors are now known to regulate the expression of the *ASS* gene such as hormones, nutrients or proinflammatory cytokines. Taken together, all the results obtained demonstrate that the factors involved act in opposite ways when considering hepatocytes or the other cells and tissues, as summarized in Table 1. The only one exception concerns cAMP that induces *ASS* gene expression in the liver [74] as well as in kidneys [112] and NO-producing cells [140,141]. Despite the physiological importance of the enzyme in various metabolic processes, little is known at a molecular level including DNA sequences and nuclear factors involved, as described below.

ASS, a known but poorly understood gene

First cloned in 1981 from human carcinoma cells [142], the *ASS* cDNA sequence was then specified for human [37], rat [39], bovine [38] and mouse [40], showing a remarkable conservation between species. Yeast and bacterial sequences were also determined [143] and, particularly, the DNA sequences of archaeobacteria, although deprived of introns, were 38% identical to that of the human gene [144], suggesting a common ancestral gene. Concerning humans, the *ASS* gene was localized on chromosome 9 [145,146] but analysis of human genomic DNA showed the presence of 14 processed dispersed pseudogenes localized on 11 chromosomes, including chromosomes X and Y [147,148]. Such pseudogenes were also identified in higher apes and rodents [40,149]. The human and murine genes span a 63-kb region and are composed of 16 exons [40]. Analysis of the mRNA in primate tissues revealed an alternative splicing [150] resulting in the presence or in the absence of exon 2 without altering the coding sequence. The biological significance of such an alternative splicing is not yet understood since exon 2 is always present in murine tissues, mostly present in the baboon liver but not in human tissues [40,150]. Moreover, two species of mRNA were observed in human cells [134,151]: a major form of about 1.7 kb and another one of about 2.7 kb which differed in the length of the 3′-untranslated region, suggesting a second polyadenylation site [152]. Again, the biological significance of the two liver mRNAs is not yet understood. Moreover, a very recent study reports the existence of three transcriptional initiation sites within exon 1 in bovine endothelial cells, resulting in 5′-untranslated region diversity of the *ASS* mRNA. This might be linked to the differential and tissue specific expression of the gene [153].

ASS, an unusual promoter

The promoter region of both human and murine *ASS* gene has been characterized partially [40,154,155]. Concerning the human gene, the 5′-flanking sequence was characterized on about 800 bp [154] showing a TATA box, six potential Sp1 binding sites (GC boxes) [154,155] and one potential AP-2 binding site [40], as illustrated in Fig. 7. Concerning the functionality of the potential binding sites, only three GC boxes have been shown acting synergistically to obtain full activation of the promoter, as demonstrated by studies on Sp1–DNA interaction [155].

Unexpectedly, no CCAAT sequence (C/EBP binding site) nor CRE (cAMP responsive-) nor GRE (glucocorticoid responsive-) elements were found. Thus, the mechanism by which hormones are acting remains totally unexplained. However, some promoter function studies and mutant mice models focused on the involvement of CREBP and C/EBPα, respectively. Firstly, a genetic locus *Tse-1*, tissue-specific extinguisher 1, that encodes the regulatory subunit R1α of PKA [156], has been shown to be responsible for the hepatic repression of several genes including the *ASS* gene in hepatoma cell/fibroblast hybrids [157]. In this context, it was clearly established that CREBP was the target of *Tse-1* repression for tyrosine amino transferase and *PEPCK* genes [158,159] but this remains to be established for the *ASS* gene. Secondly, studies with mice

Table 1. Factors involved in the tissue-specific regulation of the *ASS* gene expression. +, stimulation; + +, additivity or synergism; −, inhibition; 0, no effect.

Factors	Liver	Kidney	Other tissues and cells
Hormones and messenger			
Added alone			
Glucocorticoids	+ [58,60,63,64,67–70,74,81]	0 [110,112]	0 [111] or + [141]
Glucagon	+ [58,71,79,80]		
cAMP analogs	+ [58,71,74,75]	+ [112]	+ [140,141]
Insulin	− [79]		
Growth hormone	− [68,81,83]		0 [83]
Combined			
Glucocorticoid + glucagon	+ + [58,60,65,71–73,75,76]		
Glucocorticoid + cAMP analog	+ + [58,65,71,74,75]		+ + [141]
Glucocorticoid + insulin	0 [60,67,77]		
Glucocorticoid + GH	0 [81]		
Nutrients			
Protein diet	+ [74,84,85,88]	0 [113]	
Starvation	+ [84,85,113]	+ [112]	
Glutamine	+ [90,91]		− [13,130]
Arginine			− [131,132,135,172]
Fatty acids	− [94,96]		
Immunostimulants			
Added alone			
LPS	− [139] or 0 [118,119]	+ [118]	+ [20,118,119,121,123]
IL-1β			+ [17]
IFN-γ			+ [20]
Combined			
Cytokines[a]			+ or + + [17,120,122,128]
LPS + cytokines			+ or + + [14,18,21,121,123,124,126]
Cytokines + glucocorticoid			0 [128]
LPS + INFg + glucocorticoid			0 [18]
Others			
NH_4Cl	0 [91]		+ [136]
TGFβ			+ [137]
Shear stress			+ [138]

[a] Cytokines are different combinations of IL-1β and/or IFNγ and/or TNFα.

homozygous for deletions overlapping the albino locus on chromosome 7 (see ASS, a key step in arginine production, above), that present a decreased rate of transcription of liver *ASS* gene, focused on *alf*, a positive regulatory factor, involving C/EBPα in the regulation of gene expression [160]. The lethal locus encodes an enzyme involved in tyrosine metabolism but the mechanistic link with unrelated genes, like *ASS*, was not shown [161]. Finally, it was shown recently that *C/EBPα*-knockout mice present liver function disorders including reduced ureagenesis. In these mice, the *ASS* mRNA level was decreased and a change in the intrahepatic zonation of the *ASS* mRNA occurred [162] (see also ASS, a ubiquitous enzyme, above). This therefore suggested that C/EBPα might play a role in the regulation of the *ASS* gene expression, but the molecular mechanism is not yet established. This was not observed in *C/EBPβ*-knockout mice [163]. Concerning the action of amino acids, Sp1 was recently shown to be involved in the response to amino acid deprivation of the asparagine synthetase gene [164] and binding of this factor might eventually explain the *ASS* gene regulation by arginine or glutamine. This remains however, to be demonstrated.

We therefore performed a computer search [165] for the transcriptional factor binding sites using the published human *ASS* promoter sequence [154,155], as shown in Fig. 7. The search showed only two of the three functional Sp1 binding sites described previously [155] but one putative NF-κB site was revealed, and the functionality of this sequence remains to be proved for its involvement in the effect of cytokines on the *ASS* gene. Beside Sp1, some other transcription factors, namely HNF1, ATF2, ATF4 and C/EBPβ were involved in amino acid responses [166–169] but their binding sites were not identified by our computer search. Moreover, the following sequences 5′-ATTGCA TCA-3′ and 5′-CATGATG-3′ were identified previously as amino acid response elements (AARE) [170,171], but the specific search for these motifs on the *ASS* promoter sequence also gave negative results. Although such sequences may be localized far apart from the proximal promoter or in intragenic regions, construction of minigenes, with only the

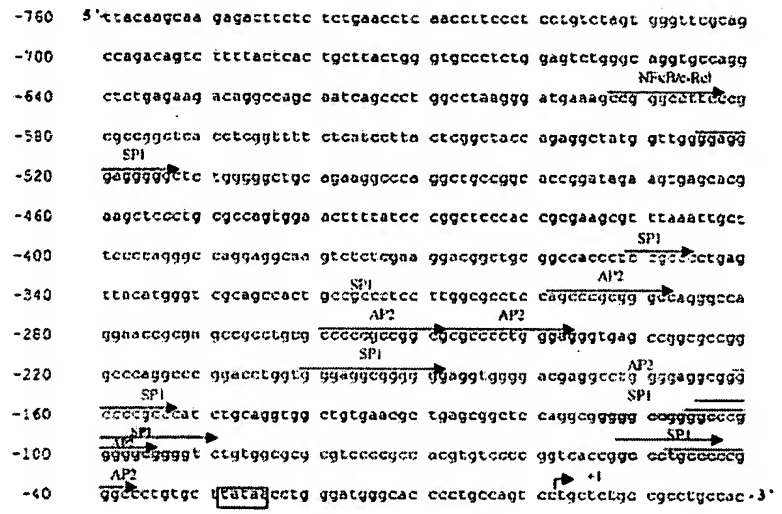

Fig. 7. Computer research for potential binding sites on the 5′-flanking sequence of the human *ASS* gene. The 5′-flanking sequence of the human *ASS* gene published by Jinno *et al.* [154] was used. The numbers indicate positions with respect to the nucleotides sequence. The TATAA consensus sequence is boxed and the transcription start site is designated (+ 1). The previously described binding sites (cumulative data from [40,154,155]) are in red type (Six Sp1 sites and one AP2 site; the three functional Sp1 sites previously analysed [40] are specified in bold type). Potential sites found in this computer research are indicated by blue arrows.The computer search of potential transcriptional factor binding sites [154] was performed using Matinspector software [165]. With the selected parameters of matching (0.8 for the core and optimized for the matrix), the analysis revealed the presence of a number of potential sites for binding with factors involved in cellular growth and differentiation (not shown). Additionally, six Sp1 binding sites were revealed, including two of the three published functional sites [40], five potential AP-2 sites, non including the identified site [40], and one potential site for NF-κB/c-REL, as shown on the sequence. This latter potential site could appear with parameters of 1 for the core and 0946 for the matrix. The missing functional Sp1 site and potential AP-2 site previously identified [40] were only found in further analysis of the sequence when parameters of matching were 0.760 and 0.976 for the core and 0.857 and 0.830 for the matrix, respectively.

first 149 base pairs of the 5′-flanking sequence of the *ASS* gene, suggested that this region contained some element(s) involved in the arginine regulation [172].

ASS, a model for gene therapy

ASS deficiency in human causes citrullinemia (see Introduction) and the classic neonatal CTLN1-form of the disease frequently leads to neonatal death [3]. This stimulated the development of gene-transfer strategies ≈ 20 years ago [173,174]. Using retroviral vectors, long-term expression of the human enzyme was obtained in mice receiving bone marrow [175], and by administration of an adenoviral vector expressing human ASS, partial correction of the enzyme defect was observed in a neonatal bovine model of citrullinemia [176]. More recently, the recombinant adenovirus transfection strategy allowed a greatly prolonged life span in a murine model of the disease [177,178]. Thus, it was suggested that, beside liver transplantation [179,180], *ASS* gene therapy might appear in the future as a potential alternative for citrullinemic patients.

Concluding remarks

Starting 50 years ago from a specific liver expressed gene, acquired knowledge has now led to recognize ASS as a ubiquitous enzyme. During this period, the physiological roles of ASS have been clearly established in different tissues and cells. Indeed, besides its key role in liver urea synthesis, it is now shown that the enzyme may play a limiting role in arginine synthesis for NO production. Moreover, the factors involved in the regulation of ASS have been identified, including hormones, nutrients and pro-inflammatory stimuli, and they were shown to act mainly at a transcriptional level. Intriguingly, however, only one transcription factor, Sp1, has been proved to interact with the *ASS* gene promoter and no clear link with the regulating molecules has been made. Moreover, regulating factors such as growth hormone, glutamine or LPS for example, may or may not regulate the *ASS* gene expression depending on the localization and the physiological role of the enzyme, i.e. urea synthesis or NO production. Thus, we still have much to learn about the molecular mechanism involved in the regulation of *ASS* gene expression and we hope this review will provide stimuli for further work.

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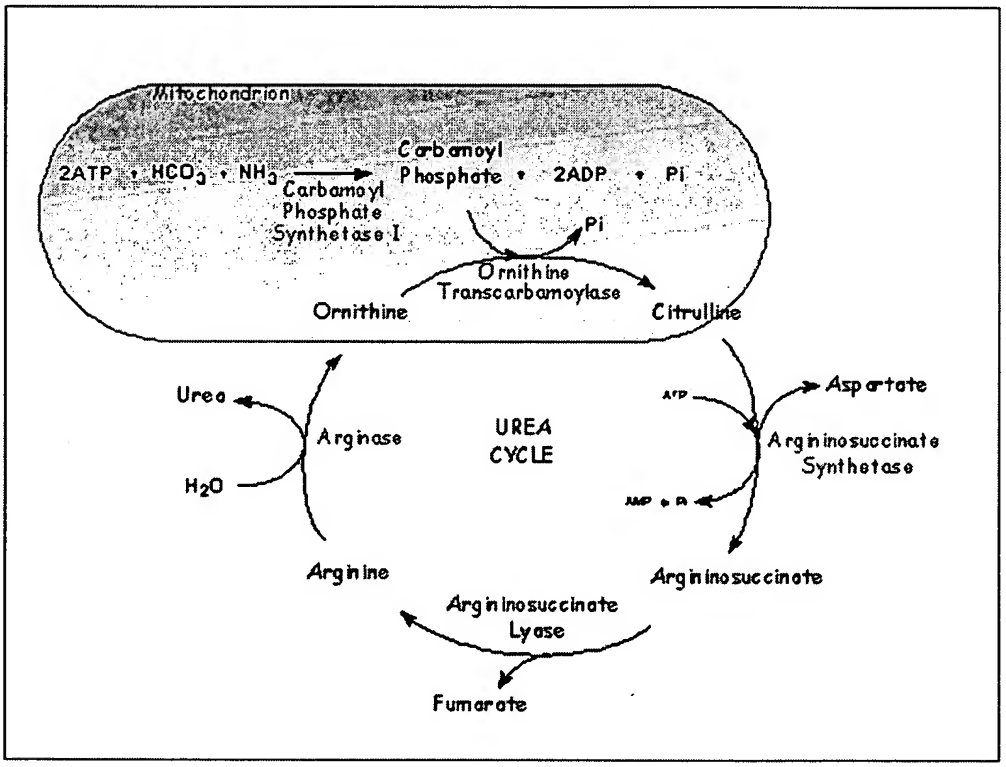
SickKids **Howell Lab research**

Arginine-citrulline/Urea cycle

The primary physiological role of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) is in the urea cycl 1). AS and AL along with nitric oxide synthase (NOS) also form the arginine-citurulline cycle, an abbreviated urea cycle non-hepatic cells, which is responsible for the overproduction of the key cell-signaling molecule, nitric oxide (NO). While production is beneficial for host defense, eliciting bactericidal, fungicidal and tumoricidal responses, its overproduction b inducible NO synthase causes septic and cytokine-induced circulatory shock. The rate-limiting factor in the synthesis of the availability of cellular arginine, and although possible sources of cellular arginine include uptake from plasma and intracellular protein degradation, the preferred source is its *de novo* biosynthesis from citrulline by argininosuccinate syr (AS) and argininosuccinate lyase (AL). We are therefore targeting AS and AL, as inhibition of either (or both) these enzy would reduce arginine formation and hence potentially prevent the overproduction of harmful NO.



(1) Argininosuccinate synthetase

AS catalyzes the first step in the production of arginine from citrulline, namely the ATP dependent ligation of citrulline ar aspartate to form argininosuccinate. We have determined the structure of *E. coli* AS at 1.6 Å resolution (Lemke and Hov Structure, 2001) and its complexes with (a) aspartate, (b) aspartate and citrulline, (c) MgATP and (d) citrulline and MgA (Lemke and Howell, JBC 2002). Our uncomplexed structure revealed that the each monomer of this tetrameric protein h structural domains: a nucleotide binding domain, similar to other N-type pyrophosphatases and a novel catalytic domair structures of AS complexed with various combinations of its substrates has allowed us to define the active site of the en and has shown that ATP binds to the nucleotide binding domain in two distinct conformations. A small conformation cha observed when citrulline and ATP bind. Since the phosphate of ATP and ureido oxygen of citrulline, which forms a cova bond during catalysis, are 5.5 Å apart, we have hypothesized that a large rigid body domain motion must occur during c

We have proposed a detailed catalytic mechanism, which is currently being tested.

(2) Argininosuccinate lyase and δ-crystallin

Argininosuccinate lyase (AL) catalyzes the second step in the production of arginine from citrulline, namely the reversibl cleavage of argininosuccinate to produce arginine and fumarate. We have two different projects on this enzyme. The fir

to understanding the catalytic mechanism of the protein, while the second seeks to understand the phenomenon of intra complementation, a phenomenon that disease causing AL mutants exhibit.

(i) Catalytic mechanism:

We are using δ crystallin, the major eye lens protein in birds and reptiles, as a model system to determine the catalytic mechanism of AL/δ2 crystallin. δ crystallin is directly related to AL by a process called gene sharing. The ancestral gene appears to have undergone a modification of gene expression to over express AL in the eye lens, where subsequent ge duplication produced two proteins, δ1 and δ2 crystallin. The δ2 protein has retained lyase activity and is the AL ortholog lens tissues, while the δ1 protein has evolved and is no longer enzymatically active. As there are only 27 amino acid dif between the δ1 and δ2 crystallin isoforms and all residues implicated directly in catalysis are conserved, this system off unique opportunity to study the enzymatic mechanism of AL/δ2 crystallin and the effect that both long-range and small conformational changes can have on catalytic activity.

During the last seven years we have determined the structures of human AL (Turner et al, PNAS, 1997) and its Q286R (Sampaleanu, Vallee, Thompson and Howell Biochemistry 2001), wild-type duck δ1 and δ2 crystallin (Sampaleanu et al Biochemistry 2001), the partially active H91N δ2 crystallin mutant, (Abu Abed et al., Biochemistry, 1997) as well as the i mutants H162N (Vallée et al, Biochemistry, 1999) and S283A (Sampaleanu et al, J. Biol. Chem. 2002) with substrate complexed. These structural studies have been complemented by biochemical studies aimed at elucidating the enzyma mechanism using site directed mutagenesis (Chakraborty et al, Biochemistry, 1999) and (Sampaleanu et al, J. Biol. Che 2002) and domain exchange experiments to recover AL activity in δ1 crystallin (Sampaleanu et al, Protein Science, 199 Combined, our research has enabled us to propose a detailed catalytic mechanism. We have mapped the residues invc substrate binding and have shown that even small perturbations in the complex network of protein-substrate interaction have a significant effect on catalysis. We have shown that it's the first structural domain of the protein that is critical for recovering AL activity in δ1 crystallin. The structures of δ1 and δ2 crystallin have also allowed us to propose that a majc conformational change in residues 270-290 and a rigid body movement of domain 3 occurs during catalysis. These conformational changes would sequester the substrate from the solvent during catalysis and suggest that S283 may be catalytic acid.

We are currently testing our proposed catalytic mechanism and have begun to extend our research to another superfan member, adenylosuccinate lyase.

(ii) Intragenic complementation

Intragenic complementation is a phenomenon that occurs when a multimeric protein is formed from subunits produced f different mutant alleles of the same gene. In a patient, complementation will ameliorate the phenotype of the disease, a residual activity of the hybrid protein will be greater than the average of the two individual mutant proteins due to their complementation. Although all genetic diseases involving multimeric proteins are subject to this phenomenon, it is often overlooked when trying to establish the genotype-phenotype relationships of a disease because the mechanisms by wh complementation occur are not well understood.

Using AL as a model system we have shown that at least two different mechanisms exist (Yu et al, Biochemistry, 2002) first mechanism, two stable active site mutants can complement to recover ~25% wild-type activity through the regener native active sites. The second mechanism involves mutations outside the active site region. In this case these typically destabilizing mutations can complement with a stable active site mutant to form a hybrid protein with increased stability partial recovery of AL activity. Two unstable mutants cannot complement to recover AL activity.

We are currently examining how mutations at the dimer and tetramer interface complement and how the protein disass and re-associates to form the hybrid proteins.